EXHIBIT 1

JB FYI BOMS

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Bacterial Genes Involved in the Elicitation of Hypersensitive Response and Pathogenesis

Intensive molecular genetic studies undertaken in the past 10 years have started to solve many of the puzzles in the area of compatibility and incompatibility between plants and bacterial pathogens. These studies have provided answers to some of the most fundamental questions in plant pathology: What bacterial genes are involved in the establishment of compatibility or incompatibility between plants and necrogenic bacteria? What traits distinguish plant-pathogenic bacteria from saprophytic bacteria? Are these genes and traits common in seemingly very diverse groups of plant-pathogenic bacteria, from soft-rot erwinias to local lesion-forming pseudomonads? In this article, we will discuss some recent advances in understanding the compatibility or incompatibility between plants and necrogenic bacteria (bacteria that cause tissue necrosis). The potential application of these advances to disease management will be addressed briefly. Interested readers should consult other recent reviews (6,8,45,50) for a more technical discussion on this topic.

Plant–Bacteria Interactions: Incompatible vs. Compatible

Plant-pathogenic bacteria cause devastating diseases on many important crop plants. Some bacteria, such as *Agrobacterium tumefaciens*, cause tissue deformation (tumors) by altering hormone balance in infected plant tissues. Other bacteria cause wilt or soft rot by interfering with the function of the plant vascular system or by disintegrating plant tissues, respectively. Many pathovars of *Pseudomonas syringae* and *Xanthomonas campestris* cause local lesions on various plant tissues. Disease symptoms caused by most plant-pathogenic bacteria involve plant cell death. In this article, only necrogenic bacteria will be discussed. Therefore, gall-forming *A. tumefaciens* and other bacteria that do not cause necrosis will not be addressed.

Plant–bacteria interactions can be generally classified as compatible or incompatible interactions. In a compatible interaction, a susceptible host plant is infected by a virulent (or compatible) bacterium, resulting in the multiplication and spread of the bacterium in infected plant tissues and the appearance of disease symptoms. In an incompatible interaction, an avirulent (or incompatible) bacterium attempts to infect a resistant host plant or a nonhost plant, but the multiplication and spread of the bacterium are severely restricted. A hallmark of many incompatible interactions is the occurrence of rapid plant cell death at or near the attempted infection site, a phenomenon known as the hypersensitive response (HR; 16,29). That is, although an avirulent bacterium is unable to cause typical spreading disease symptoms in a resistant host or nonhost plant, it is able to elicit localized plant cell death. The HR is associated with a wide array of defense responses that may inhibit further pathogen invasion, including synthesis of antimicrobial compounds, induction of plant defense genes, and strengthening of the plant cell wall by rapid cross-linking of cell wall components (10,32).

Although a true plant-pathogenic bacterium can elicit a dramatic plant response—either disease or resistance—in a healthy plant with the appropriate genetic background, saprophytic bacteria or bacteria that are pathogenic on organisms other than higher plants are generally unable to initiate any interactions in plants. Of 1,600 known species of bacteria, only about 80 species have been found to cause plant disease (1). What are the features that distinguish plant pathogenic bacteria from other types of bacteria? Taxonomic differences give no clue to the differences in pathogenicity. For example, *Erwinia amylovora*, the bacterium that causes fire blight, is taxonomically more closely related to the human pathogens *Escherichia coli* and *Yersinia* spp. than to another common plant pathogen, *P. syringae*.

Genes Controlling Compatibility Between Plants and Bacteria

In the early 1980s, a number of researchers started to use transposon-mediated mutagenesis, a technique developed in the study of *E. coli*, to reveal bacterial genes that play important roles in various plant–bacteria interactions. A transposon is a mobile DNA element that can hop in and out of the bacterial chromosome. When a transposon hops into a gene on the chromosome, the gene is physically disrupted and cannot produce a functional product (Fig. 1). If the gene happens to be important in plant–bacterial interactions, the mutant bacterium carrying the disrupted gene will show a defect in initiating normal plant–bacterial interactions.

Using such a mutagenesis technique, Niepold et al. (35) and Lindgren et al. (33) identified clusters of bacterial genes, known as *hrp* (for HR and pathogenicity) genes, in the bean pathogens *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *phaseolicola*, respectively. Transposon-induced mutations in *hrp* genes were found to abolish the ability of *P. syringae* to elicit the HR in nonhost plants or to cause disease in host plants (33,35). *hrp* mutants behave very much like bacteria that have no apparent interactions with plants, such as *E. coli*. The identification of *hrp* genes suggested that the molecular mechanism(s) underlying bacterial pathogenicity and bacterial elicitation of plant disease resistance may involve the same bacterial genes.

hrp genes have been isolated from many plant-pathogenic bacteria and have been characterized most extensively from *P. s.* pv. *syringae*, *P. s.* pv. *phaseolicola*, *Pseudomonas solanacearum* (which causes wilt in many solanaceous plants), *Xanthomonas campestris* pv. *vesicatoria* (which causes bacterial spot on tomato and pepper), and *E. amylovora* (6,8,45). Isolation (cloning) of *hrp* genes was accomplished by inserting random genomic DNA fragments from a wild-type, plant-pathogenic bacterium into a cloning vector, followed by introduction of cloned DNA fragments into *hrp* mutants

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(Fig. 1). If a cloned DNA fragment carries a wild-type copy of the mutated *hrp* gene in an *hrp* mutant, it will produce a functional *hrp* gene product and therefore complement the mutated *hrp* gene located in the chromosome (Fig. 1). Surprisingly, the cloned *hrp* clusters from *P. s.* pv. *syringae* 61 and *E. amylovora* 321 enabled nonpathogens (e.g., *E. coli* or *Pseudomonas fluorescens*) to elicit the HR in plants (5,24). The functional cloning of these two *hrp* clusters in *E. coli* revealed that the minimum number of genes required for elicitation of the HR by plant-pathogenic bacteria is carried on a DNA fragment about 25 to 30 kb in length, a very small portion of the bacterial genome (which is normally about 4,000 to 5,000 kb).

DNA–DNA hybridization studies indicate that at least some *hrp* genes are similar among necrogenic bacteria belonging to different genera (*P. syringae*, *E. amylovora*, *Erwinia stewartii*, *P. solanacearum*, and *X. campestris*) (31). Recent DNA sequence studies confirm that many *hrp* genes cloned from diverse plant-pathogenic bacteria are homologous (23,46). Thus, *hrp* genes appear to be universal among diverse necrosis-causing, gram-negative bacterial pathogens of plants.

Biochemical Functions of *hrp* Genes

The biochemical functions of *hrp* genes have remained a puzzle until recently. DNA sequencing has played a major role in the determination of many *hrp* gene functions. As will be discussed, many *hrp* genes have striking similarities with genes of known function. Figure 2 shows the gene organization and likely functions of *hrp* genes of *P. s.* pv. *syringae* (23). There are at least 25 *hrp* genes in this bacterium. Based on DNA sequence similarity to other known genes and subsequent biochemical and molecular characterization, we now know that *hrp* genes have at least three biochemical functions: gene regulation, protein secretion, and production of HR elicitor proteins.

1. Gene regulation. It was discovered that *hrp* genes either are not expressed or are expressed at very low levels (i.e., they make very low levels of protein products) when bacteria were grown in nutrient rich bacteriological media, whereas they are highly expressed when bacteria are in the intercellular space (apoplast) of plant tissues (25,37,41,46,48,52,53). What conditions in plant tissues induce the expression of *hrp* genes, and how do bacteria detect these inducing conditions? Unlike viruses, nematodes, and many fungi, plant-pathogenic bacteria do not invade living plant cells. Therefore, signal exchanges between plant cells and bacteria must occur in (or through) the apoplast outside the plant cell. A number of laboratories have observed that induction of *P. syringae hrp* genes could be achieved by using artificial minimal media lacking complex nitrogen nutrients, indicating that lack of nutrients in the plant apoplast may be the signal for the induction of *hrp* genes (25,37,52,53). Specific compounds (e.g., sucrose and sulfur-containing amino acids) present in the plant apoplast may also serve as signals for the induction of *X. c.* pv. *vesicatoria hrp* genes (41). The induction of *hrp* genes in the nutrient-poor plant apoplast or in artificial minimal media indicates that *hrp* genes may be involved in bacterial nutrition in planta.

How do bacteria sense the plant apoplast environment? It was found that at least three of the 25 *hrp* gene products are involved in detection of the apoplast environment by *P. syringae*: *hrpL*, *hrpS*, and *hrpR* (18,51; Fig. 2). The *hrpS* and *hrpR* are among the first two *hrp* genes to be expressed once bacteria enter plant tissues (51,52). It has been hypothesized that the HrpS and HrpR proteins, once produced, bind to the "promoter" sequence of the *hrpL* gene to "promote" the production of the *HrpL* protein (51). Once the HrpL protein is produced, it activates promoters of other *hrp* genes and some bacterial avirulence (*avr*) genes, which determine gene-for-gene interactions between bacteria and plants (25,26,38,40,51; Fig. 3). Not all bacterial *avr* genes are regulated by *hrp* genes (30). Interestingly, *hrpS* and *hrpR* are similar in sequence to a family of bacterial proteins that regulate genes involved in diverse metabolic functions, including genes involved in nutrient transport and metabolism (18,51). The sequence similarity of *hrpS* and *hrpR* with gene regulators involved in nutrition appears to support the hypothesis that *hrp* genes are involved in bacterial nutrition in the nutrient-poor plant apoplast. This hypothesis is further supported by the observation that the expression of *hrp* genes can be turned off by complex nitrogen sources, tricarboxylic acid cycle intermediates, high osmolarity, and neutral pH, some of which are characteristic of rich bacterial media (25,37,41,46,52,53).

An *hrpS* homolog has been found in a very different bacterium, *E. amylovora* (S. V. Beer, *personal communication*). In *P. solanacearum*, a different *hrp* gene (*hrpB*) was found to be involved in the detection of the plant apoplast (15). Thus, different bacteria may or may not use the same mechanism to detect the apparently similar environment in the plant apoplast.

2. Protein secretion. One surprising finding from the sequence analysis of *hrp* genes was that many *hrp* genes show striking similarities to those involved in the secretion of proteinaceous virulence factors in human and animal pathogenic bacteria (12,17,22,39,46). Most plant-pathogenic

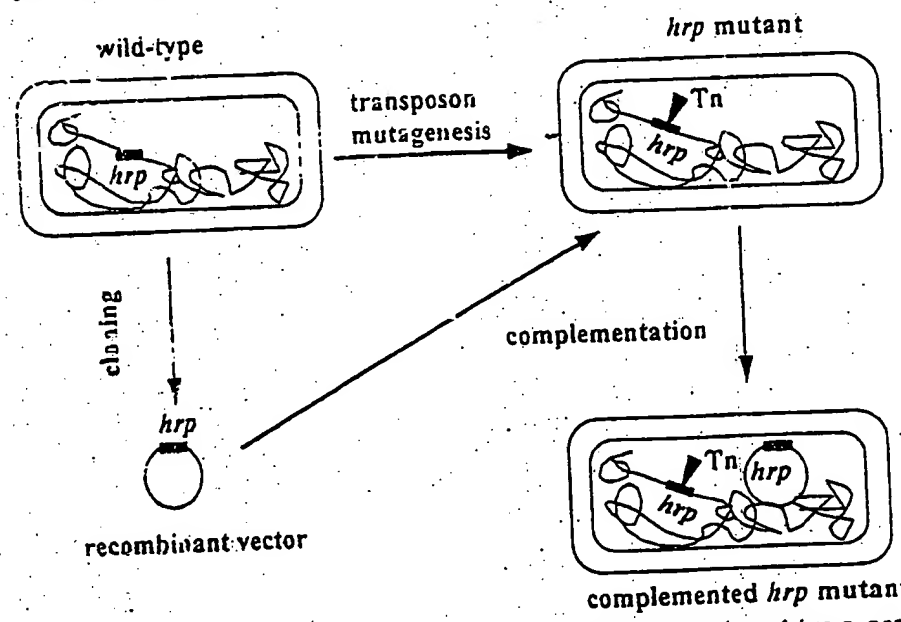

Fig. 1. Diagram of molecular techniques commonly used in the cloning of *hrp* genes. A wild-type bacterium is mutagenized by random insertion of a transposon (Tn) into its genome. When a transposon inserts into a wild-type *hrp* gene (in red), it physically disrupts the *hrp* gene (in green). The transposon-inserted *hrp* gene cannot produce a functional product, and the resulting bacterium is called a *hrp* mutant. The *hrp* mutant cannot longer induce the hypersensitive response (HR) in resistant plants or cause disease in susceptible plants. To isolate (clone) the *hrp* gene identified by transposon mutagenesis, a gene library is established by inserting pieces of the wild-type genome DNA into a cloning vector (indicated by a circle). The vector carrying foreign DNA inserts (recombinant vector) is then introduced into the *hrp* mutant. If a recombinant vector happens to carry a wild-type copy of the mutated *hrp* gene, it will produce a functional *hrp* gene product lacking in the *hrp* mutant, thus recovering the ability of the mutant to induce the HR in resistant plants and to cause disease in susceptible plants. The *hrp* mutant phenotype is therefore complemented by this recombinant vector.

bacteria that cause necrosis are gram-negative, that is, they have two cell membranes enveloping the cytoplasm. For any large molecule (e.g., a protein) to go through a lipid membrane, a special secretion apparatus or channel composed of many proteins must be assembled across both cell membranes. Gram-negative plant pathogenic bacteria are known to make several types of secretion apparatus. For example, *Erwinia chrysanthemi*, a bacterium that causes soft rot, makes one type of secretion apparatus for proteases and another for plant cell wall–degrading enzymes (21,39). Both types of secretion apparatus are widely conserved among many other bacteria, including human pathogens such as *E. coli* and *Pseudomonas aeruginosa* (21,39). The *hrp* genes were found to specify a third type of secretion apparatus, the Hrp secretion apparatus, which appears to be similar to the one discovered in several human-pathogenic bacteria, including *Yersinia* spp. (12,17,22,39,46). Interestingly, although the regulatory *hrp* genes in different bacteria may be different (*hrpS*, *hrpR*, and *hrpL* in *P. syringae* versus *hrpB* in *P. solanacearum*), most *hrp* genes involved in the assembly of the Hrp secretion apparatus are similar among diverse plant-pathogenic bacteria. This suggests that although different bacteria may detect the plant apoplast environment in their own unique ways, they nevertheless produce similar types of protein secretion apparatus.

3. **Production of elicitor proteins.** The discovery of the novel Hrp secretion apparatus raised an immediate question: What are the proteins that traverse it? Since *hrp* genes are essential for bacteria, both to elicit the plant HR and to cause disease, it was expected that some of the proteins that traverse the Hrp secretion apparatus may be elicitors of plant HR and that others may be involved in causing necrosis during pathogenesis. Wei et al. (47) first provided evidence that one of the *E. amylovora hrp* genes (*hrpN*) encodes a proteinaceous elicitor (harpin). Harpin elicits HR necrosis when injected into the apoplast of appropriate plants (47). Although no *hrpN* gene homolog could be found in *P. syringae*, another proteinaceous HR elicitor ($harpin_{Pss}$) was identified and was shown to be encoded by a different *hrp* gene, *hrpZ* (20,36). Furthermore, $harpin_{Pss}$ was the first extracellular protein shown to be secreted via the Hrp secretion apparatus (20). A third bacterial protein elicitor of the HR was identified in *P. solanacearum* and was named PopA1 (2). The *E. amylovora* harpin, *P. s.* pv. *syringae* 61 $harpin_{Pss}$, and *P. solanacearum* PopA1, although largely dissimilar in primary sequences, share similar properties that may be important in their HR elicitor activities. They are all heat stable, glycine rich, and hydrophilic. Homologs of *E. amylovora* harpin and *P. s.* pv. *syringae* 61 $harpin_{Pss}$ have been identified in other pathogenic strains that belong to the genus *Erwinia* and the species *P. syringae*, respectively (4,20). Thus, at least three proteins that traverse the Hrp secretion apparatus of three diverse bacteria elicit the HR.

The Search for Proteins that Traverse the Hrp Apparatus

As mentioned earlier, bacterial mutants defective in the Hrp secretion apparatus are unable to elicit the HR in resistant plants and to cause disease in susceptible plants. The question is, how many proteins are secreted via the Hrp secretion apparatus? If harpins and PopA are the only proteins that traverse the Hrp secretion apparatus, then mutations in the genes that make harpins and PopA would also eliminate the ability of bacteria to elicit the HR in resistant plants and to cause disease in host plants. However, if there are other pathogenicity- or HR-related proteins secreted via the Hrp apparatus, mutations in only harpin- or PopA-encoding genes would not completely abolish the ability of bacteria to elicit the HR in resistant plants or to cause disease in host plants. Wei et al. (47) reported that mutations in the gene coding for harpin of *E. amylovora* destroyed the ability of the bacteria both to trigger the HR in resistant nonhost tobacco and to cause disease in susceptible pear fruits. Mutations in the gene coding for $harpin_{Ech}$ of *E. chrysanthemi* prevented the bacterium from triggering the HR in the nonhost tobacco and reduced the ability of the bacterium to initiate disease lesions in host plants (4). In the case of $harpin_{Pss}$ of *P. syringae*, mutation analysis has been complicated by the complex gene structure and organization surrounding the *hrpZ* gene. Conclusive data regarding the role of $harpin_{Pss}$ in plant–*P. syringae* interactions are yet to be shown. PopA1 was shown to

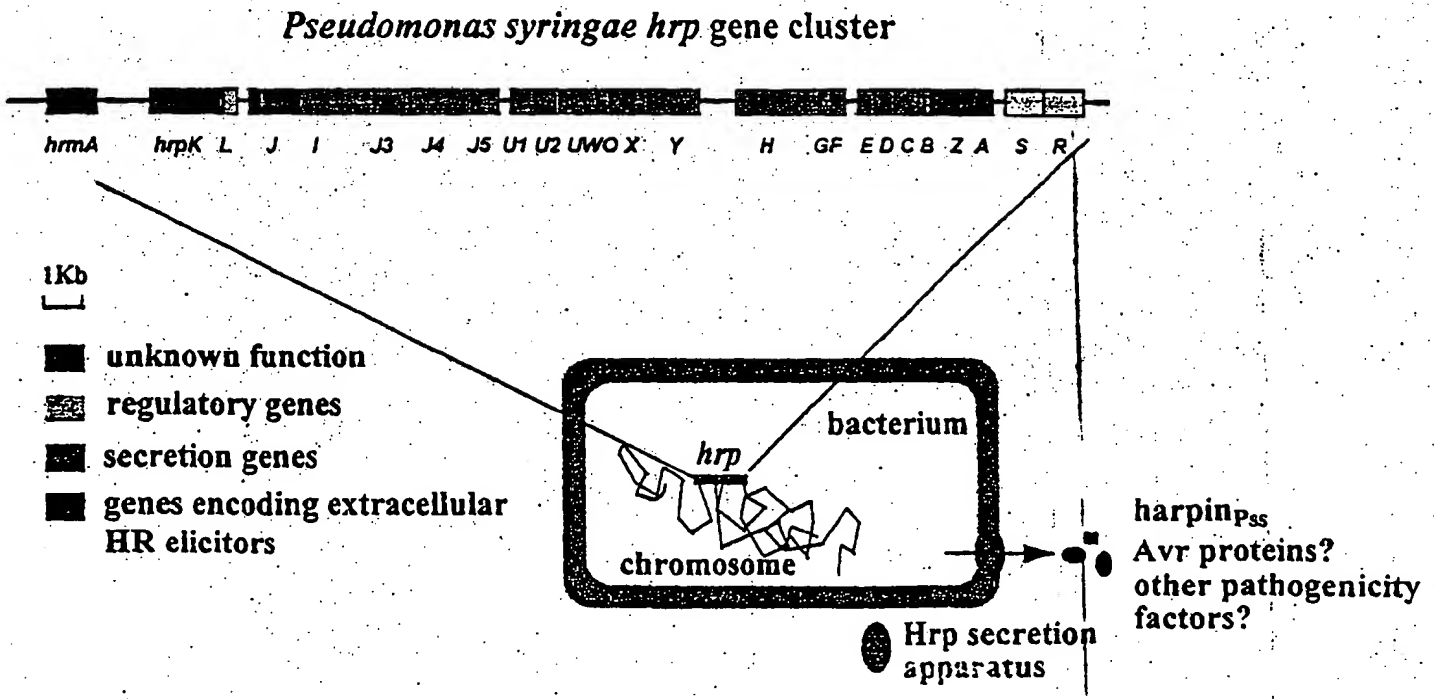

Fig. 2. *hrp* genes of *Pseudomonas syringae*. There are at least 25 *hrp* genes (*hrpA* to *hrpZ*) in *P. syringae*. *hrpS*, *hrpR*, and *hrpL* (in yellow) are involved in the detection of the plant apoplast environment and in the activation of all other *hrp* genes, *avr* genes, and possibly other pathogenicity-related genes. Most other *hrp* genes (in red) are involved in the assembly of the Hrp secretion apparatus in the bacterial envelope, through which travels a newly discovered class of bacterial virulence/avirulence proteins (in green), including HrpZ.

be dispensable for pathogenicity of *P. solanacearum* in the susceptible host plant, tomato, or for HR elicitation in the nonhost plant, tobacco (2), indicating that there must be other HR-elicitors and pathogenicity factors that traverse the Hrp secretion apparatus in this bacterium. Further examination indicated that PopA1 may function as an avirulence gene, mediating gene-for-gene interaction in certain Petunia–*P. solanacearum* interactions (2,45). Thus, the Hrp secretion apparatus in each bacterium may secrete a different number of proteins. Identification of other proteins that traverse the Hrp secretion apparatus is now an active research area and is essential for a complete understanding of *hrp*-mediated plant–bacterial interactions.

Factors Modifying *hrp* Gene-Mediated Compatibility

Two broad classes of bacterial genes may superimpose their functions on the *hrp* gene-mediated compatibility or incompatibility between plants and bacteria: *avr* genes and various virulence genes. The *avr* genes mediate genotype-specific incompatibility in resistant host plants. Virulence genes promote the production of disease symptoms and are usually needed for the full virulence of bacteria.

Bacterial *avr* Genes

Flor (14) formulated the gene-for-gene hypothesis in his work on flax–flax rust interactions. Flor hypothesized that the resistance of a given flax cultivar to a given fungal race is the result of the interaction between a fungal *avr* gene and a corresponding flax resistance gene. Therefore, the interactions between the plant's resistance genes and the pathogen's *avr* genes would limit the host range of the pathogen. Staskawicz et al. (44) first cloned an *avr* gene from a soybean bacterial pathogen, *Pseudomonas syringae* pv. *glycinea*, and showed that the cloned *avr* gene could convert virulent *P. s.* pv. *glycinea* strains that cause disease into avirulent strains that elicit the HR in certain soybean cultivars carrying the corresponding resistance genes, thus validating the role of *avr* genes in controlling host range. Since then, numerous *avr* genes have been cloned from plant-pathogenic bacteria (27). Several plant resistance genes have also been cloned using molecular genetic approaches (e.g., 34,43).

What is the relationship between the *avr* genes and *hrp* genes, both of which are involved in eliciting the HR? Several laboratories have observed that *avr* genes cannot trigger the genotype-specific HR in *hrp* mutants, i.e., *avr* genes depend on functional *hrp* genes for expressing their phenotype (25,26,28,38,40). There are several ways of explaining such dependence (Fig. 4). One possibility is that Avr proteins are dependent on the Hrp secretion apparatus for secretion. Alternatively, Avr function requires a prior plant response elicited by the *hrp*-controlled extracellular factors (such as harpins). A third possibility is that Avr proteins, with no HR-eliciting activity by themselves, cause the cultivar-specific HR by either covalently modifying harpins or modulating the expression of harpins in a plant resistance gene–dependent manner yet to be understood. Finally, it is also possible that Avr proteins are secreted directly into the plant cell with the help of harpins, assuming that receptors for Avr proteins are inside the plant cell. Studies are being carried out to resolve these possibilities.

Bacterial Virulence Factors

The genetic diversity of plant-pathogenic bacteria is reflected in their ability to cause diverse disease symptoms ranging from soft rot to tissue necrosis to "wildfire." These diverse disease symptoms are likely the result of the action of several, sometimes unique, virulence factors produced by a given bacterium in addition to *hrp*-controlled pathogenicity factors. For example, research from many laboratories has shown that toxin production plays an important role in the formation of chlorosis and necrosis (3,19,49). Extracellular polysaccharides may be involved in the formation of water-soaking lesions (11,13) and in the production of wilt symptoms by clogging the plant vascular system (9). Plant cell wall–degrading enzymes are responsible for tissue disintegration and the appearance of the soft-rot symptom (7). Plant hormones produced by plant-pathogenic bacteria are involved in the induction of tissue deformation (42).

Both *hrp* genes and bacterial virulence factors are necessary for disease symptom production, but what is the relationship between them? A logical relationship would be that *hrp*-controlled extracellular factors are involved in obtaining nutrients in early stages of pathogenesis, whereas other virulence factors drive the initial compatible stage into a fully compatible one, leading to the production of various disease symptoms. At least two lines of

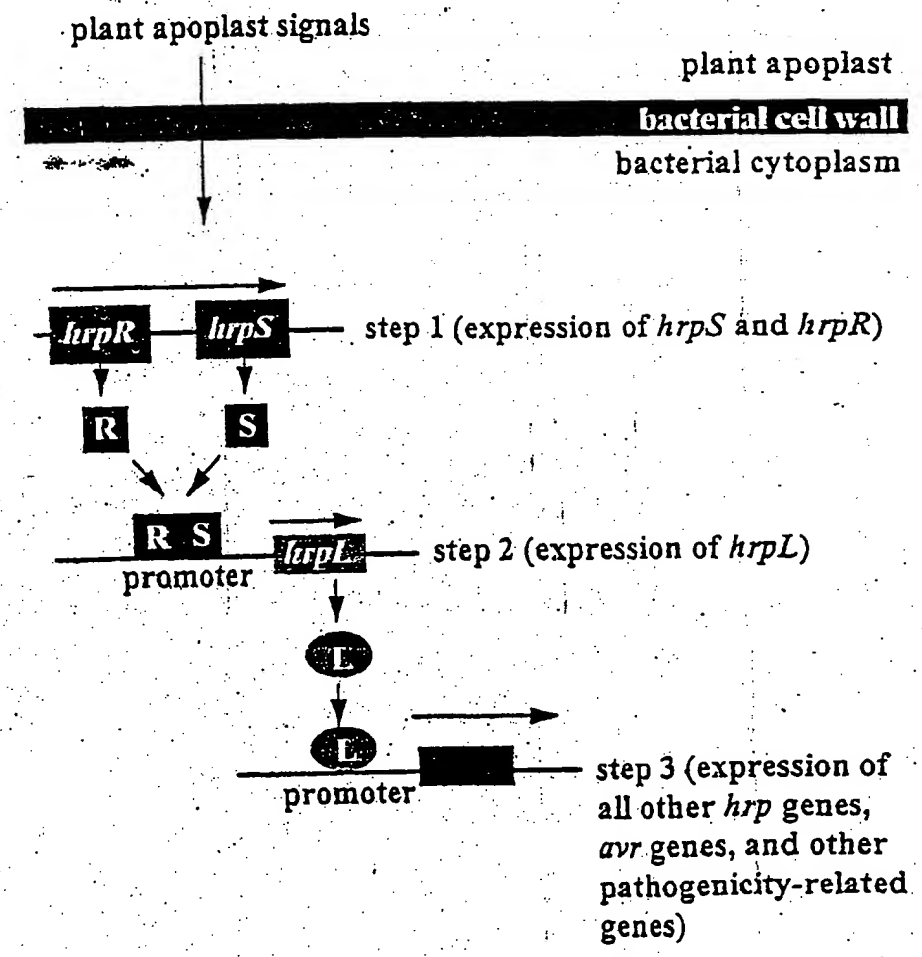

Fig. 3. Diagram of the signal transduction cascade in the detection of the plant apoplast environment by *Pseudomonas syringae*. The plant apoplast environment (limited nutrients and/or certain unique compounds) activates the expression of *hrpS* and *hrpR* by a mechanism yet to be understood (step 1). The *hrpS* and *hrpR* gene products (S and R, respectively) bind to and activate the promoter of the *hrpL* gene (step 2). The *hrpL* gene product (L), in turn, binds to promoters of other *hrp* genes, *avr* genes, and other bacterial pathogenicity-related genes to promote the expression of these genes, resulting in the initiation of diverse plant–bacteria interactions (step 3). Modified from Xiao et al. (51).

evidence seem to support this relationship. First, *hrp* genes are highly conserved among diverse plant-pathogenic bacteria, whereas virulence factors vary greatly among bacteria. Second, while mutations in the *hrp* gene completely abolish both bacterial pathogenicity and elicitation of the HR, mutations in virulence genes (e.g., toxin-production genes) often do not eliminate pathogenicity and have no effect on bacterial elicitation of the HR (3,19,49).

hrp Gene Functions and Disease Management

A major reason for discovering bacterial and plant factors critical for bacterial pathogenesis and plant resistance is to develop novel and environmentally safe strategies for controlling plant diseases. The discovery that the Hrp secretion apparatus is crucial to bacterial pathogenesis provides a foundation for designing novel chemicals and antibodies that would block the assembly of the Hrp secretion apparatus or the passage of bacterial virulence proteins through it. Alternatively, susceptible crop plants could be genetically engineered with genes encoding proteinaceous HR elicitors, such as harpins, under the control of plant promoters inducible by virulent pathogens. If this approach were successful, the HR or resistance would be triggered in otherwise compatible interactions, limiting disease development.

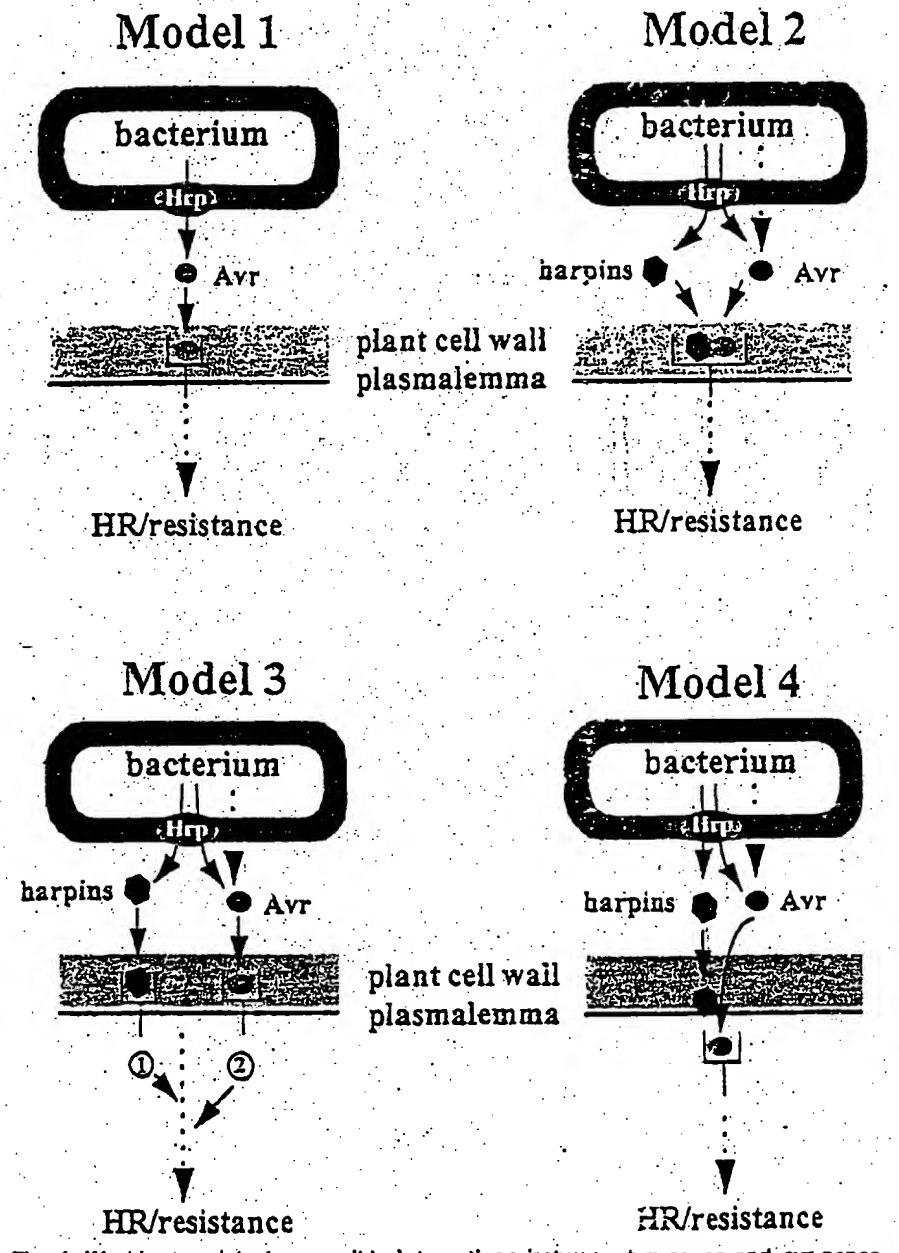

Fig. 4. Working models for possibl interactions between *hrp* genes and *avr* genes. Model 1: Avr signals (Avr proteins or their enzymatic products) are secreted through the Hrp secreti n apparatus to elicit the hypersensitiv response (HR) and resistance. Model 2: Harpins and Avr signals modify each other before interacting with plant receptors. Avr signals may or may not be secreted via the Hrp secretion apparatus. Model 3: Harpins and Avr signals interact with respective plant receptors. Plant response elicited by harpins must precede plant resp ns elicited by Avr signals. Avr signals may or may not be secreted via the Hrp secretion apparatus. Model 4: Avr proteins are secreted int the plant cell with the help of harpins. Avr signals may or may not be secreted via the Hrp secretion apparatus. In m dels 1 to 3, receptors for Avr pr teins are presumed t be on the plant cell surface. In model 4, receptors for Avr proteins are inside the plant cell.

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EXHIBIT 2

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Bacterial home goal by harpins

Ulla Bonas

Host–pathogen interactions are dynamic and multifactorial; whether a microorganism succeeds or fails in colonizing a potential host depends on factors from both organisms. A successful pathogen has to overcome the defenses of the host. In bacteria that are pathogenic for animals or for plants, particularly Gram-negative organisms, a large number of genes are essential to infect host tissue and establish disease. Expression of these genes is generally controlled by environmental conditions such as temperature, pH, salt concentration and nutrient availability[1,2].

Pathogenicity, hypersensitive reaction and elicitors

In the Gram-negative plant pathogens *Erwinia*, *Pseudomonas* and *Xanthomonas*, genes organized in clusters of 25–40 kb are fundamentally involved in any obvious interaction with a plant (for a review see Ref. 3). These genes have been designated *hrp* (hypersensitive reaction and pathogenicity) because they are essential not only for pathogenicity towards a susceptible host plant, but also for interaction with resistant host varieties and with plants that are not a host for that pathogen. In plants, the hypersensitive reaction (HR) (Ref. 4) is a rapid defense reaction involving localized plant cell death and production of substances such as phenolics and phytoalexins at the site of infection. The HR prevents pathogen spread and thus halts disease development.

In the wild, plants are resistant to the majority of pathogens. The HR, therefore, is an important defense mechanism against all kinds of possible disease agents (bacteria, fungi, nematodes and viruses). It is not only important to interactions of pathogens with nonhost plants, but also to interactions between plants that carry resistance genes and microorganisms that are pathogens for that species.

Although the genes involved in plant defense[5,6] are becoming better understood, very little is known about the nature of the initial signals and their perception. Induction of the HR in a bacterium–plant interaction requires functional *hrp* genes and appears to be mediated by signal molecules or 'elicitors'. Recent DNA sequence analyses indicate that several putative Hrp proteins from different species are related and may be involved in a secretion system reminiscent of secretion of Yops (*Yersinia* outer proteins) in *Yersinia*[7–11]. So far, only one specific elicitor of the HR in a bacterium–plant interaction has been described. The *avrD* gene from *Pseudomonas syringae* pv. *tomato* mediates production of a low-molecular-mass compound that specifically induces the HR only in the soybean plant (a nonhost) when it carries the corresponding *Rpg4* resistance gene[5,12].

Harpins

Recently, two bacterial HR-inducing proteins, called 'harpins', were identified in *Erwinia amylovora*[13] and *P. syringae* pv. *syringae*[14]. Although the harpins differ in primary sequence, they have several features in common: they are glycine rich and heat stable, and they both induce an HR in tobacco, a nonhost plant for these bacteria. The genes encoding harpins are localized within the *hrp* clusters and obviously have a dual role in that they are also required for pathogenicity towards the normal host plant. Both *hrp* clusters allow nonpathogenic bacteria, such as *Escherichia coli*, to induce an HR in tobacco after recombinant expression, suggesting that the genes for the tobacco HR elicitors are present within the clusters[15,16].

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The first harpin to be identified, harpin$_{Ea}$, is a cell-envelope-associated protein encoded by the *hrpN* gene of *Er. amylovora*, a pathogen of pear and apple[13]. Recently, He and co-workers[14] have used an elegant approach to identify harpin$_{Pss}$, which is encoded by the *hrpZ* gene in the bean pathogen *P. s.* pv. *syringae*. Lysates of an expression library in *E. coli*, made using the cloned *P. s.* pv. *syringae hrp* cluster, were directly screened for HR-inducing activity on tobacco leaves. Two proteins were identified, one of which was an amino-terminal deletion of harpin$_{Pss}$ with even higher activity than the full-size protein; whether processing occurs during natural infection is not clear. Interestingly, the carboxyl terminus contains two short, direct repeats that are essential for elicitor activity. The activity is in the same range as that of the *Erwinia* harpin$_{Ea}$; however, to elicit an HR in other plants requires higher levels of the elicitor. He *et al.* show convincingly that the secretion of harpin$_{Pss}$ by *P. s.* pv. *syringae* depends on a product called HrpH that is closely related to proteins in other plant pathogens, and also in animal pathogens such as *Yersinia* and *Shigella*, where they are essential for protein secretion[9,10,14].

These exciting findings help verify the model that Hrp proteins are involved in the transport of elicitors and virulence factors[7]. Not surprisingly, the results presented by He and co-workers[14] also stimulate many questions. It needs to be shown that harpin$_{Pss}$ is actually secreted when the bacterium interacts with tobacco tissue (the *hrp* genes were induced *in vitro*). The concentration needed for HR induction (more than 600 nM) is much higher than one would expect for specific signal molecules. Are harpins toxins? Most importantly, what is their function in pathogenicity, and why do they

not elicit an HR in the host plant? Are harpins the only elicitors of nonhost HR in tobacco and possibly in other plants? Is the same mechanism used in tobacco to recognize both the *Erwinia* and the *P. s.* pv. *syringae* harpins? Is host resistance different in mechanism from nonhost resistance? Answers to this fascinating puzzle require the identification of more HR elicitors and their putative plant receptors.

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Initiation and spread of α-herpesvirus infections

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Herpesviruses are large animal viruses with a DNA genome varying from approximately 120 to 250 kb. Based on their biological properties, the Herpesviridae have been divided into three subfamilies, the α-, β- and γ-herpesvirinae, prototypes of which are the human pathogens herpes simplex virus (HSV), cytomegalovirus (HCMV) and Epstein–Barr virus (EBV), respectively. As enveloped viruses, they depend on two consecutive processes for infectious entry into target cells: (1) attachment of free virions to cells and (2) penetration, that is, fusion of virion envelope and cellular cytoplasmic membrane leading to release of the nucleocapsid into the cell. Virion envelope glycoproteins play important roles in both these processes (see Refs 1,2 for recent reviews).

After infection of primary target cells, virus spread can occur by several different mechanisms. Infected cells may release infectious virions that reinitiate infection from outside. In addition, direct viral cell-to-cell spread from primary infected cells to adjacent noninfected cells may occur. In the host, virus may be disseminated by circulating infected cells that adhere to noninfected tissues and transmit infectivity directly. Recent results on HSV and pseudorabies virus (PrV) shed more light on these processes in α-herpesviruses. PrV causes Aujeszky's disease in swine, which is characterized by nervous and respiratory symptoms, and reproductive failure. Unlike HSV, PrV is not pathogenic for humans. However, the two viruses have several features in common, including a broad host range *in vitro*, and several species besides the natural host can be infected experimentally. In addition, all of the known PrV glycoproteins are related to homologous glycoproteins in HSV (Ref. 1)*.

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Attachment

Binding of free infectious virus to target cells involves interactions between virion envelope glycoproteins and cellular virus receptors. Herpes virions contain a large number of different virus-encoded envelope glycoproteins that might participate in attachment. A well-known example of a cellular herpesvirus receptor is the B-cell membrane protein CR2 (CD21), which binds EBV (Ref. 3). Recent studies have demonstrated that several α- (reviewed in Ref. 1), β- and γ-herpesviruses[4,5] bind to their target cells by interaction of virion components with cell-surface glycosaminoglycans, principally heparan sulfate (HS)[6].

*At the 18th International Herpesvirus Workshop, a common nomenclature for α-herpesvirus glycoproteins was agreed on, based on designations of HSV glycoproteins. This nomenclature is used here.

EXHIBIT 3

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hrp Genes of Phytopathogenic Bacteria

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Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Pages 79-98 in: Current Topics in Microbiology and Immunology, Vol. 192: Bacterial Pathogenesis of Plants and Animals - Molecular and Cellular Mechanisms. J. L. Dangl, ed. Springer-Verlag, Berlin.

1 Introduction

In nature plants are resistant to the majority of pathogens, and many bacteria live in close contact with the plant without causing any harm (see chapter by Beattie and Lindow in this volume). Among the 1600 different species known in the bacterial kingdom only a small number (about 80) are plant pathogenic and in most cases highly specialized with respect to the plant that can be attacked. Only a few of these species are gram-positive, e.g., *Clavibacter* ssp. and *Streptomyces* ssp. In this review I focus on subspecies of the gram-negative genera *Erwinia, Pseudomonas,* and *Xanthomonas,* which comprise the major bacterial plant pathogens.

To be a successful pathogen the invading bacterium has to overcome the plant's defense. During evolution plant pathogenic bacteria have acquired multiple functions that enable them to colonize and multiply in living plant tissue. In nature, bacteria enter the plant through natural openings (stomata, hydathodes) or

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wounds. The bacterial armory contains a number of weapons that contribute to pathogenicity. Obvious examples include degradative extracellular enzymes such as pectinases, cellulases, and proteases. When the corresponding genes are mutated, bacterial ability to invade plant tissues is more or less affected depending on the pathogen, i.e., these functions contribute to and modulate development and severity of infection to different extents (see chapters by Dow and Daniels, and Collmer and Bauer in this volume).

In addition, phytopathogenic bacteria possess a large number of genes needed for basic pathogenicity. These genes have been operationally defined as *hrp* (hypersensitive reaction and pathogenicity; LINDGREN et al. 1986) based on their mutant phenotype. *hrp* genes are not only essential for pathogenicity on a plant, i.e., the ability to cause disease in a compatible interaction, but also for the incompatible interaction with resistant host varieties or with plants that are not normally a host for the particular pathogen (so called non-host). The incompatible interaction is often associated with the induction of a hypersensitive reaction (HR) in the plant. In contrast to the use of the term hypersensitivity in the animal field, in plants the HR is a rapid defense response involving localized plant cell death, production of phenolics and antimicrobial agents, e.g., phytoalexins, at the site of infection (KLÉMENT 1982; LINDSAY et al. 1993). The HR results in prevention of pathogen multiplication and spread and thus in prevention of disease development. Under natural infection conditions the HR is microscopically small and can be induced by just one bacterial cell. Only when bacteria are introduced into plant tissue at high cell densities in the laboratory (about 10^7 colony forming units or more/ml) is the HR macroscopically visible as confluent necrosis and can be clearly distinguished from typical disease symptoms. It is important to note that saprophytic or nonpathogenic bacteria such as *Escherichia coli* or *Pseudomonas fluorescens* do not induce the HR and are unable to multiply in plant tissue.

2 Isolation of *hrp* Genes and General Features

hrp genes have been isolated from all major gram-negative plant pathogenic bacteria except Agrobacterium. There are excellent reviews that describe the early work or focus more on one particular pathogen (WILLIS et al. 1991; BOUCHER et al. 1992). The majority of *hrp* genes have been identified by complementation of loss-of-function mutants. Mutants obtained by random chemical (e.g., N-methyl-*N'*-nitro-*N*-nitrosoguanidine) or transposon mutagenesis of a pathogenic wild-type strain were inoculated into the host plant and screened for loss of both the ability to cause disease in susceptible plants and to induce the HR in resistant host or non-host plants (often tobacco). The second criterion for the isolation of genes specific for the plant interaction was to ensure that the mutants would still grow in minimal medium. This way mutants affected in genes for basic housekeeping functions were eliminated. A third characteristic of all *hrp* mutants is that they are unable to grow in the plant.

The *hrp* genes were originally described for the bean pathogen *Pseudomonas syringae* pv. *phaseolicola*. LINDGREN and coworkers (1986) isolated Tn*5*-induced mutants of *P.s.* pv. *phaseolicola* that had lost both the ability to induce halo-blight disease on bean and the HR in tobacco. Complementation with cosmid clones from a genomic library of the wild-type strain resulted in isolation of a cluster of *hrp* genes localized in a 20 kb DNA region. This was the first indication that both the ability to cause disease and to induce the HR are mediated by common steps in a "pathway".

Since then *hrp* gene clusters have been cloned from a number of different bacteria. Examples include *Pseudomonas solanacearum* (BOUCHER et al. 1987; Fig. 1B), the *Xanthomonas campestris* pathovars *campestris* and *vitians* (ARLAT et al. 1991), *translucens* (WANEY et al. 1991), and *vesicatoria* (BONAS et al. 1991; Fig. 1A), *Erwinia amylovora* (STEINBERGER and BEER 1988; BARNY et al. 1990; WALTERS et al. 1990; BAUER and BEER 1991), and several other pathovars of *P. syringae* (e.g., HUANG et al. 1988; LINDGREN et al. 1988; Fig. 1C). In addition, genes with DNA homology, and in some cases functional homology, have been isolated from other species, e.g., the so-called *wts* genes from *E. stewartii* (COPLIN et al. 1992; LABY and BEER 1992), and a region containing pathogenicity genes from *X.c.* pv. *glycines* that complement *hrp* mutants of *X.c.* pv. *vesicatoria* (HWANG et al. 1992; Bonas, unpublished results). Interestingly, nonpathogenic xanthomonads that were originally isolated from diseased plants as opportunists together with pathogenic bacteria do not contain *hrp*-related DNA sequences (STALL and MINSAVAGE 1990; BONAS et al. 1991). In *Agrobacterium tumefaciens* or in strains of *Rhizobium* ssp. there seem to be no *hrp* gene equivalents present (BONAS et al. 1991; LABY and BEER 1992). This conclusion is based on DNA hybridization experiments and, of course, does not exclude the presence of genes with functional homology to *hrp* genes in these species.

In all of the cases mentioned above, the *hrp* genes are organized in clusters of 22–40 kb, and I will restrict most of this chapter to these large *hrp* clusters. In addition, several smaller *hrp* loci have been described that are not linked to the large cluster present in the same bacterium. These include a region in *P. solanacearum* (HUANG et al. 1990), the *hrpX* locus that is conserved in *X. campestris* pathovars *campestris* (KAMOUN and KADO 1990; KAMOUN et al. 1992) and *oryzae* (KAMDAR et al. 1993), and the *hrpM* locus in *P.s.* pv. *syringae* (NIEPOLD et al. 1985; MUKHOPADHYAY et al. 1988). *hrpM* is functionally conserved in pathovar *phaseolicola* (FELLAY et al. 1991). Besides being nonpathogenic and unable to induce the HR in tobacco, *P. syringae hrpM* mutuants are also affected in mucus production. The *hrpM* locus encodes two putative proteins which are similar and have been shown to be functionally homologous to the products of the *E. coli mdoGH* operon (LOUBENS et al. 1993). The *mdoGH* genes are required for periplasmic membrane-derived oligosaccharide synthesis in *E. coli*. The *hrpQ* and *hrpT* genes from *P.s.* pv. *phaseolicola* (MILLER et al. 1993) will be discussed later in this chapter.

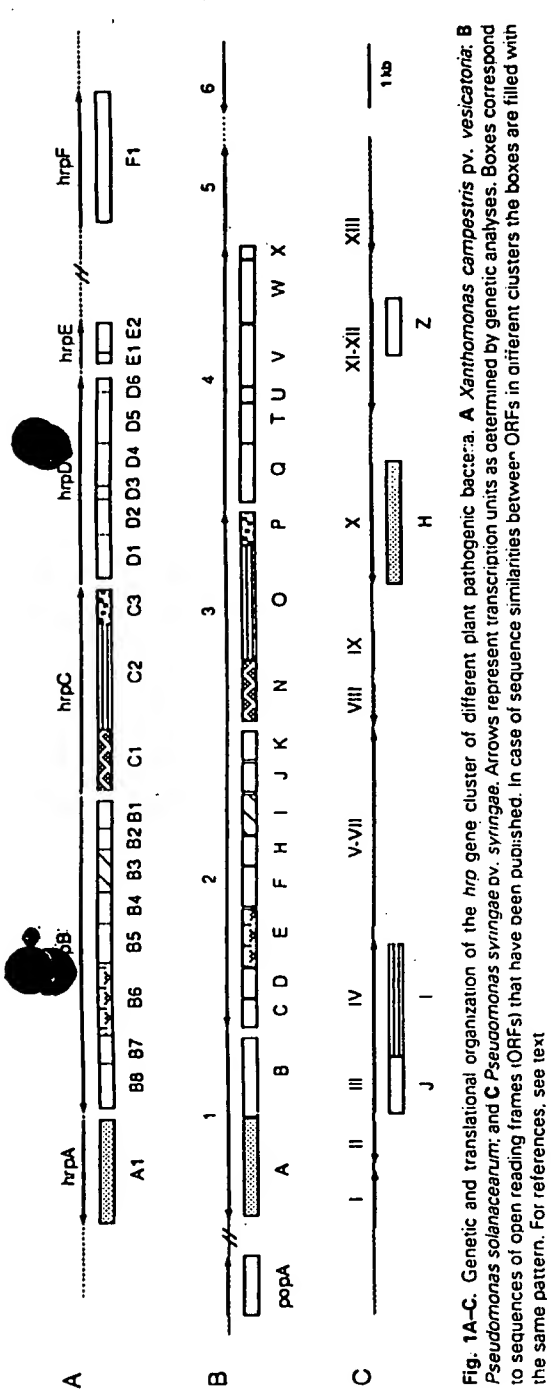

Fig. 1A–C. Genetic and translational organization of the *hrp* gene cluster of different plant pathogenic bacteria. **A** *Xanthomonas campestris* pv. *vesicatoria*; **B** *Pseudomonas solanacearum*; and **C** *Pseudomonas syringae* pv. *syringae*. Arrows represent transcription units as determined by genetic analyses. Boxes correspond to sequences of open reading frames (ORFs) that have been published. In case of sequence similarities between ORFs in different clusters the boxes are filled with the same pattern. For references, see text

3 Structural Organizati n and Relatedness f *hrp* Clusters

Genetic studies using transposon-induced insertion mutants in the respective bacterial wild-type strains revealed that the *hrp* clusters contain at least six to eight complementation groups (Fig. 1). Some *hrp* gene clusters have clearly been shown to be localized in the chromosome, e.g., in *P.s.* pv. *phaseolicola* (RAHME et al. 1991) and in *X.c.* pv. *vesicatoria* (BONAS et al. 1991), whereas in *P. solanacearum*, the *hrp* cluster is on a megaplasmid (BOUCHER et al. 1987).

Striking similarities have recently been found between the *hrp* genes of pathogens belonging to different genera. The first indication of homologies came from Southern hybridization studies. DNA homology was observed among different strains of the same pathovar, as well as between pathovars or strains within a species, and in some cases also between species. However, the degree of conservation varies. DNA homology is high within pathovars of a given subspecies, e.g., in *P. syringae* (LINDGREN et al. 1988; HUANG et al. 1991) and in *X. campestris* (BONAS et al. 1991). The latter studies were recently extended by PCR using primers based on *hrp* sequences from *X.c.* pv. *vesicatoria* (LEITE et al. 1994). Furthermore, at least some regions of the *hrp* clusters appear to be conserved on the DNA level between *P. solanacearum* and pathovars of *X. campestris*, *P. syringae*, and also to *E. amylovora* (BOUCHER et al. 1987; ARLAT et al. 1991; GOUGH et al. 1992; LABY and BEER 1992). In addition, cross-complementation within a subspecies indicated a high degree of functional conservation of *hrp* genes (e.g., LINDGREN et al. 1988; ARLAT et al. 1991; BONAS et al. 1991; LABY and BEER 1992). Due to sequence data it is now becoming more and more apparent that several *hrp* genes are conserved in all major gram-negative plant pathogenic bacteria (see below). Whether there are *hrp* genes that are clearly pathovar-specific can only be answered when complete sequence information becomes available for several *hrp* clusters.

4 Function of *hrp* Genes in *Xanthomonas campestris* pv. *vesicatoria* and Other Plant Pathogenic Bacteria

DNA sequence analysis of the *hrp* genes has revealed some important clues to their possible biochemical functions. One of the first genes sequenced was a regulatory gene, *hrpS*, from *P.s.* pv. *phaseolicola* (GRIMM and PANOPOULOS 1989). This gene as well as *hrpB*, a regulatory gene from *P. solanacearum* (GENIN et al. 1992), will be discussed below in the context of gene regulation.

Since *hrp* genes are environmentally regulated (see below), it was believed for a while that they would be encoding "alternative" proteins required for adaptation of the bacterium to the plant as the preferred environment. The recently discovered sequence similarities between several putative Hrp proteins and known proteins from other bacteria, however, led to a very different hypothesis, namely, involvement of Hrp proteins in protein secretion. We have

sequenced the entire *hrp* cluster of *X.c.* pv. *vesicatoria*. Since most *hrp* sequences from this and other bacteria are not yet published, I will summarize our results and refer to the other phytopathogenic bacteria as I go along. Based on genetic analyses and the open reading frames (ORFs) with a high coding probability we predict 21 *hrp* genes in the 25 kb *hrp* cluster of *X.c.* pv. *vesicatoria*. Their transcriptional organization is depicted in Fig.1A. The loci *hrpA* and *hrpB* are transcribed from right to left; the other four loci are transcribed from left to right (SCHULTE and BONAS 1992a). According to the locus (*hrpA–hrpF*) we have numbered the ORFs consecutively. The *hrpA* locus appears to contain just one *hrp* gene, *hrpA1*. The *hrpB* operon contains eight ORFs, called *hrpB1–hrpB8*, etc. A region of about 4 kb between *hrpE* and *hrpF* does not seem to be involved in the interaction with the plant because insertions in this region do not lead to a change in phenotype (BONAS et al. 1991).

What are the characteristics of the Hrp proteins? It should be noted that, except for three proteins, expression of the other 18 has yet to be demonstrated in *X.c.* pv. *vesicatoria*. A number of putative Hrp proteins are most likely associated with or localized in the bacterial membrane. For example, the HrpC2 protein sequence contains eight transmembrane domains but lacks a signal sequence, suggesting an inner membrane localization (FENSELAU et al. 1992). Both HrpA1 and HrpB3 contain an NH_2-terminal signal sequence and one (HrpA1) or two (HrpB3) transmembrane domains, suggesting that a part of these proteins might be targeted to the outer membrane. The signal sequence of HrpB3 resembles signal peptidase II sequences which are typical of lipoproteins (FENSELAU et al. 1992). Experiments using radioactively labeled palmitate are underway to test whether HrpB3 is a lipoprotein. Recently, both HrpB3 and HrpA1 were shown to be localized in the *X.c.* pv. *vesicatoria* membrane fraction using polyclonal antibodies (S. Fenselau, C. Marie, and U. Bonas, manuscript in preparation). The HrpB6 protein is a putative ATPase with highly conserved nucleotide and magnesium binding domains. It is more similar to protein traffic ATPases than to proton pump ATPases, and the lack of membrane spanning domains suggests a cytoplasmic location (FENSELAU et al. 1992).

Searches of the database revealed sequence relatedness of more than half of the *X.c.* pv. *vesicatoria* Hrp proteins with putative proteins in other bacteria, including different plant pathogens. High DNA sequence identity (more than 90%) was found to a 2.7 kb fragment carrying pathogenicity genes from *X.c.* pv. *glycines* (HWANG et al. 1992). The authors predicted two ORFs, whereas in *X.c.* pv. *vesicatoria*, this region contains three ORFs corresponding to the *hrpC3*, *hrpD1* and *hrpD2* genes. Complementation studies indicated that part of the *hrp* region is colinear in the two pathovars of *Xanthomonas* (unpublished).

The deduced amino acid sequences of *hrp* genes published from *P. solanacearum* (GOUGH et al. 1992, 1993; GENIN et al. 1992) show significant similarity to *X.c.* pv. *vesicatoria* proteins (Table 1; Fig.1). One exception is the *hrpB* regulatory gene from *P. solanacearum* which is not present in the 25 kb *hrp* region or in the flanking region of the *X.c.* pv. *vesicatoria hrp* cluster as determined by DNA sequence analysis and hybridization studies (T. Horns and U. Bonas, unpublished). Furthermore, several of the proteins mentioned are conserved in other species (Fig.1), however, the degree of sequence similarity varies greatly (Table 1). The HrpA1 protein from *X.c.* pv. *vesicatoria* is 48% and 29% identical to proteins from *P. solanacearum* (HrpA; GOUGH et al. 1992) and *P.s.* pv. *syringae* (HrpH; HUANG et al. 1992), respectively. HrpC2 from *X.c.* pv. *vesicatoria* is even more conserved, being 66% idential to the corresponding HrpO protein of *P. solanacearum* (GOUGH et al. 1993), whereas the *hrpI* genes from *E. amylovora* (WEI and BEER 1994) and from *P.s.* pv. *syringae* (HUANG et al. 1993) both show 62% similarity to *hrpC2* from *X.c.* pv. *vesicatoria*. *P.s.* pv. *syringae* also contains a *hrpB3* related gene, called *hrpY*, and a *hrpD2* related gene, *hrpW* (H.-C. Huang, personal communication). Thus, the high degree of DNA sequence conservation that was reported earlier (see above) is also seen on the protein level. It appears that *hrp* genes in *X.c.* pv. *vesicatoria* are more closely related to *P. solanacearum* than to *P. syringae* and to *Erwinia*. As more and more homologous *hrp* genes are found in other bacteria nomenclature might become confusing. However, as long as the genes have not been shown to be functionally homologous, we will continue to use these names.

Besides genes that are conserved among the major phytopathogenic bacteria some genes are absent in the *hrp* region of more distantly related species. For example, there are no known homologs of the harpin genes *hrpN* (WEI et al. 1992a), and *hrpZ* (HE et al. 1993) (see below), and of *hrpJ* from *P.s.* pv. *syringae* (HUANG et al. 1993) in the *X.c.* pv. *vesicatoria hrp* cluster (unpublished; see Fig. 1).

Similarities of 50%–60% were found recently between HrpA1 and HrpB3 from *X.c.* pv. *vesicatoria* and two putative NoI proteins of *Rhizobium fredii* that are encoded by a cultivar specificity region. NoIT and NoIW mutants have a wider host range in nodulation of soybean (MEINHARDT et al. 1993). In addition, the authors mention that release of proteins is affected.

Last but not least, Table 1 summarizes the significant sequence similarities which have been found to proteins from animal bacterial pathogens. A number of putative Hrp proteins are related to proteins in animal pathogens such as *Salmonella*, *Shigella*, and *Yersinia* ssp. Since the first similarities found were to the Ysc, Vir, and Lcr proteins from *Yersinia* ssp, this group of organisms became a "role model" for plant pathologists (FENSELAU et al. 1992; GOUGH et al. 1992; HUANG et al. 1992). In *Yersinia*, these proteins are essential for the secretion of virulence factors, called Yops (Yersinia outer protein; MICHIELS et al. 1990, 1991). Since they are described in detail in the chapter by G.R. Cornelis, I will mention only a few important features. The Yops are hydrophilic proteins that lack a typical NH_2-terminal signal peptide, and are secreted by using an entirely different pathway from that previously described for protein secretion. The genes involved in secretion are clustered on a 70 kb virulence plasmid. In case of a mutation, e.g., in *Ysc*, the Yops accumulate in the cytoplasm (MICHIELS et al. 1991). Although their direct role in transport has yet to be demonstrated, it is believed that the Ysc and Lcr proteins mentioned in Table 1 are parts of a special transport apparatus for Yop secretion. Similarly, *Shigella flexneri* secretes virulence factors, called Ipa (invasion plasmid antigens), that are distinct from Yops but share the general

Table 1. Sequence similarities of *Xanthomonas campestris* pv. *vesicatoria* Hrp proteins

Xanthomonas campestris pv. *vesicatoria*	HrpA1[1]	HrpB6[1]	HrpB3[1]	HrpC1[2]	HrpC2[1]	HrpC3[2]	HrpD1[2]	HrpD2[2]
Pseudomonas solanacearum	HrpA[3] (66%)	HrpE[4]	HrpI[3] (70%)	HrpN[5] (74%)	HrpO[3] (81%)	HpaP[5] (54%)	HrpQ[4]	HrpT[4]
Pseudomonas syringae pv. *syringae*	HrpH[6] (52%)				HrpI[7] (62%)			
Yersinia enterocolitica	YscC[8] (55%)		YscJ[8] (56%)					
Yersinia pestis	YscC[9] (55%)				LcrD[10] (70%)		LsaA[11] (52%)	LsaB[11] (72%)
Yersinia pseudotuberculosis		YscN[12] (73%)	LcrKa[13] (56%)					
Shigella flexneri	MxiD[14] (50%)	Spa47[15] (65%)	MxiJ[16] (52%)	Spa40[17] (55%)	MxiA[18] (65%)			Spa24[15] (67%)
Salmonella typhimurium	InvG[19] (52%)	SpaL[20] (70%) FliI[21] (65%)		SpaS[20] (56%)	InvA[22] (67%)			SpaP[20] (64%)
Bacillus subtilis		FlaA-ORF4[23] (68%)		FlhB[24] (62%)	FlhA[25] (63%)			FliP[26] (68%)
Escherichia coli		β-F1[27] (53%)						FliP[28] (65%)
Erwinia carotovora							MopB[29] (49%)	MopC[29] (65%)
Erwinia amylovora					HrpI[30] (62%)			
Rhizobium fredii	NolW[31] (51%)		NolT[31] (61%)					
Caulobacter crescentus					FlbF[32] (55%)			

Similarities between deduced amino acid sequences of Hrp proteins from *X.c.*pv.*vesicatoria* and other proteins include conservative amino acid exchanges. Number in parentheses indicates percent similarity.

Superscript numbers indicate references as follows:

1, FENSELAU et al. 1992; 2,Bonas et al., unpublished; 3, GOUGH et al. 1992; 4, GENIN et al. 1993, sequences unpublished; 5, GOUGH et al. 1993; 6, HUANG et al. 1992; 7, HUANG et al. 1993; 8, MICHIELS et al. 1991; 9, HADDIX and STRALEY 1992; 10, PLANO et al. 1991; 11, Fields et al. unpublished, accession # L22495; 12, Galyov, unpublished, accession # U00998; 13, RIMPILAINEN et al. 1992; 14, ALLAOUI et al. 1993; 15, VENKATESAN et al. 1992; 16, ALLAOUI et al. 1992; 17, SASAKAWA et al. 1993; 18, ANDREWS and MAURELLI 1992; 19, Lodge et al., unpublished, accession # X75302; 20, GROISMAN and OCHMAN 1993; 21, VOGLER et al. 1991; 22, GALÁN et al. 1992; 23, ALBERTINI et al. 1991; 24, Carpenter et al., unpublished, accession # X741212; 25, CARPENTER and ORDAL 1993; 26, BISCHOFF et al. 1992; 27, SARASTE et al. 1981; 28, MALAKOOTI et al. unpublished, accession # L21994; 29, MULHOLLAND et al. 1993; 30, WEI and BEER 1993; 31, MEINHARDT et al. 1993; 32, RAMAKRISHANAN et al. 1991; SANDERS et al. 1992.

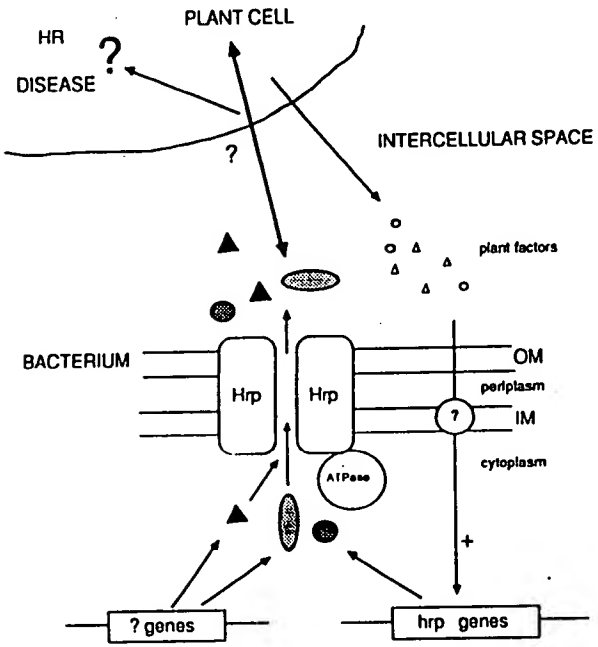

Fig. 2. Hypothetical model of cell signaling between gram-negative bacteria and plants indicating the proposed function of Hrp proteins as an apparatus for protein secretion. The model has been modified after FENSELAU et al. (1992). Hrp proteins may form a tunnel that enables the export of molecules such as virulence factors or avirluence factors leading to either a hypersensitive response (HR) or disease. These factors could be encoded by *hrp* genes or genes unlinked to the large cluster. Both types of genes have been found to encode elicitors of the HR (see text). The secretion of virulence proteins is hypothetical

features mentioned above (HALE 1991; and see chapter by PARSOT, this volume). Although *S. typhimurium* appears to possess a secretion system similar to that in *Shigella*, secreted invasion antigens have not yet been identified (GROISMAN and OCHMAN 1993; see chapter by FINLAY). As unpublished reports indicate that more and more genes in the animal pathogens are conserved, the data shown in Table 1 will soon be out of date. Proteins from other bacteria, e.g., *E. coli*, *Bacillus*, *Caulobacter* and from the *mop* region in *E. carotovora* (MULHOLLAND et al. 1993), have also been found to be similar to Hrp proteins (Table 1). Most of these are important for the assembly of the flagella, motility, or chemotaxis, again pointing, in my opinion, to a specialized secretion system rather than an involvement of *hrp* genes in chemotaxis.

These observations led us and others to propose a *hrp*-dependent secretion system in plant pathogenic bacteria (FENSELAU et al. 1992; GOUGH et al. 1992; VAN GIJSEGEM et al. 1993). A model is shown in Fig. 2 and raises certain questions, e.g., if secretion occurs, what is being secreted by plant pathogenic bacteria? So far, a few proteins have been identified as elicitors of the HR but there is no evidence for secretion of virulence factors (see below).

5 *hrp*-dependent Secretion of Hypersensitive Resp nse-Inducing Proteins

5.1 Harpin from *Erwinia amylovora*

An important feature of the isolated *hrp* clusters from both *E. amylovora* and *P.s.* pv. *syringae* is the ability of *E. coli* or *Pseudomonas fluorescens* transformants containing the cloned genes to induce the HR on tobacco (HUANG et al. 1988; BEER et al. 1991; see below). This has prompted to search for the HR-inducing activity within the respective gene clusters.

The first bacterial HR-inducing protein identified, designated harpin, is a cell envelope-associated protein encoded by the *hrpN* gene of *E. amylovora*, a pathogen of pear and apple (WEI et al. 1992a). This harpin$_{Ea}$ is a glycine-rich and heat-stable protein that induces the HR in the non-host, tobacco. The *hrpN* gene is localized within the respective *hrp* cluster and thus has a dual role in also being required for pathogenicity on the normal host plant. Its function in pathogenicity, however, is unknown. BEER et al. (1993) mentioned in a preliminary report that the *hrpN* gene seems to be conserved among *Erwinia* ssp. but that there is no DNA homology between *hrpN* and sequences in the other plant pathogenic bacteria. Although data described below suggest that the harpin$_{Ea}$ protein might be secreted via the Hrp secretory apparatus, there is no published information available that demonstrates this.

5.2 Harpin from *Pseudomonas syringae* pv. *syringae*

Using an elegant approach He and coworkers recently have identified harpin$_{Pss}$, which is encoded by the *hrpZ* gene in the bean pathogen *P.s.* pv. *syringae* (HE et al. 1993; see Fig. 1C and chapter by Collmer and Bauer). Lysates of *E. coli* clones containing an expression library, made using the cloned *P.s.* pv. *syringae hrp* cluster, were directly screened for HR-inducing activity on tobacco leaves. Two proteins were identified, one of which was an NH_2-terminal deletion of harpin$_{Pss}$ with even higher activity than the full size protein. Whether or not processing occurs in natural infection is not clear. Interestingly, two short direct repeats in the COOH-terminus of harpin$_{Pss}$ are essential for elicitor activity. Although the two harpins harpin$_{Ea}$ and harpin$_{Pss}$ differ in their primary sequence, they have several features in common, e.g., a stretch of 22 amino acid that is similar in both proteins (HE et al. 1993). Harpin$_{Pss}$ is also glycine-rich and heat-stable. As with harpin$_{Ea}$ of *E. amylovora*, the function of harpin$_{Pss}$ in pathogenicity is unknown. Its product is secreted by *P.s.* pv. *syringae* in a HrpH-dependent way; HrpH is highly related to proteins involved in secretion in other plant and animal pathogens (HUANG et al. 1992; see Table 1).

5.3 *PopA* from *Pseudomonas solanacearum*

An HR-inducing protein has been identified and characterized from *P. solanacearum* culture supernatants, called Pop (Pseudomonas out protein; ARLAT et al. 1994). PopA1 and two shorter derivatives, PopA2 and PopA3, induce the HR in tobacco and in certain, but not all, *Petunia* lines. Like the harpins, the Pop proteins are also heat-stable and glycine-rich, however, the sequence is entirely different. In contrast to the harpins, the *popA* gene is not a *hrp* gene but is located outside of the large *hrp* cluster. Interestingly, expression of *popA* is *hrpB*-dependent, i.e., the gene is part of the *hrp* regulon. Mutations in *popA* do not affect the HR on tobacco or pathogenicity on tomato suggesting that more than one HR-inducing factor is produced. ARLAT et al. (1994) convincingly showed that secretion of PopA is dependent on other *hrp* genes, such as *hrpA*, *hrpN*, and *hrpO* (Fig. 1B). If a bacterial strain virulent towards *Petunia* is found it will be interesting to see if PopA acts as an avirulence protein in *Petunia* as has been suggested by the authors.

These exciting findings prove that certain Hrp proteins of *P.s.* pv. *syringae* and *P. solanacearum* play a role in transport of HR elicitors (Fig. 2). They also stimulate more questions. It needs to be shown that harpins and PopA are in fact secreted when the bacteria interact with the plant (the *hrp* genes were induced in vitro). Are harpins conserved among pathovars of *P. syringae*? How many elicitors of the non-host HR in tobacco can be found? Is the mechanism of recognition in tobacco identical with the *Erwinia* and *P.s.* pv. *syringae* harpins and the *P. solanacearum* Pops?

6 Regulation of Expression of *hrp* Genes

Expression of *hrp* genes is controlled by environmental conditions and has been studied on the RNA level as well as using transcriptional fusions to reporter genes such as the *E. coli* genes encoding β-galactosidase or β-glucuronidase. In general, expression of *hrp* loci is not detectable when the bacteria are grown in complex culture media. However, after growth of the bacteria in the plant, *hrp* genes are expressed. Attempts to mimic the conditions that the different bacterial species encounter in the plant tissue resulted in the finding that growth in minimal media without any plant-derived factor was sufficient to induce *hrp* genes. This has led to the speculation that the bacteria have to experience some kind of starvation conditions for full expression of *hrp* genes. One of the first indications for *hrp* gene expression in vitro, and clearly a breakthrough, was a report on the *hrp*-dependent expression of an avirulence gene from the soybean pathogen *P.s.* pv. *glycinea* (HUYNH et al. 1989).

Since the composition of minimal media differs depending on the bacterium studied, the most important findings will be summarized for representative pathogens. Parameters like carbon source, concentration of organic nitrogen and phosphate, osmolarity, and pH have been found to be important. High concentration of organic nitrogen generally appears to suppress *hrp* gene activation. Only two regulatory genes have been studied so far (see below). Interestingly, they both belong to different families of regulatory proteins.

6.1 *Pseudomonas syringae*

Expression of all seven *hrp* loci in the large cluster of *P.s.* pv. *phaseolicola* is suppressed in complex medium but induced in the plant. Induction occurs in the susceptible host plant as well as in the non-host, tobacco, suggesting that there is no plant species-specific molecule involved in control of host range (RAHME et al. 1992). Five complementation groups, *hrpAB*, *hrpC*, *hrpD*, hrpE and *hrpF*, can also be induced in M9 minimal medium containing sucrose as a carbon source, however, induction is affected by pH, osmolarity, and carbon source, and never reaches the levels obtained in the plant (RAHME et al. 1992). A similar observation was made earlier for the avirulence gene *avrB* in *P.s.* pv. *glycinea*. Induction occurred in a minimal medium containing fructose, mannitol, or sucrose. Expression of *avrB* is dependent on *hrp* genes homologous to *hrpRS* and *hrpL* from *P.s.* pv. *phaseolicola* and was suppressed by TCA cycle intermediates such as citrate and succinate (HUYNH et al. 1989). *hrp* gene expression in *P.s.* pv. *syringae* occurs in the same medium as described by HUYNH et al. (1989); (HUANG et al. 1991; XIAO et al. 1992). The authors showed *hrp* gene induction in the non-host plant, tobacco, but no data for the host plant. The *P.s.* pv. *phaseolicola* loci *hrpL* and *hrpRS* are only expressed to a very low level in M9 minimal medium and are induced at least 1000-fold when the bacteria are inoculated into the plant. This led to the conclusion that, at least for expression of *hrpL* and *hrpRS*, specific plant factors might be necessary (RAHME et al. 1992).

6.2 Regulatory Genes *hrpRS* and *rpoN* of *Pseudomonas syringae* pv. *phaseolicola*

The results on environmental factors inducing or suppressing *hrp* gene expression suggested that specific regulatory genes are involved in the control of *hrp* promoter activities. At least two loci are involved in positive regulation of the other *hrp* loci of *P.s.* pv. *phaseolicola hrp* cluster (FELLAY et al. 1991). While there is no information published for *hrpL*, *hrpRS* has been sequenced. It contains two genes whose predicted protein products are 60% identical to each other (GRIMM and PANOPOULOS 1989; MILLER et al. 1993). The HrpS protein is similar to members of the NtrC family of regulatory proteins in gram-negative bacteria. Most NtrC-like regulatory proteins are members of two-component systems, with a sensor protein that in turn activates a response element by phosphorylation of a site in the conserved NH_2-terminal domain (ALBRIGHT et al. 1989). The putative sensor component operating in *hrp* gene regulation has not been identified. It is postulated that HrpS is the activating protein, however, direct biochemical data

have not been presented. The lack of a typical NH_2-terminal domain in HrpS could indicate that a different mechanism may be involved in HrpS activation. Apparently, *hrpS*-related sequences are also present in other bacteria, e.g., in *P.s.* pv. *syringae* (Heu and Hutcheson 1993) and in *Erwinia amylovora* (Beer et al. 1993). *E. stewartii* contains a transcriptional regulator, WtsA, with 52% identity to HrpS of *P.s.* pv. *phaseolicola*. The *hrpS* clone, however, was unable to functionally complement a *wtsA* mutant (Frederick et al. 1993).

The structure of the *hrpRS* locus and the finding of –24/–12 consensus sequences upstream of *hrpRS* indicated a possible role in transcriptional activation for transcription factor sigma 54, encoded by *rpoN* (Grimm and Panopoulos 1989). In a preliminary report, Felley et al. (1991) demonstrated that *hrp* gene expression in *P.s.* pv. *phaseolicola* is indeed dependent on *rpoN*. A *rpoN* mutant of *P.s.* pv. *phaseolicola* is a glutamine auxotroph and nonpathogenic. Whether *rpoN* is generally involved in regulation of *hrp* gene expression is not clear. In *X.c.* pv. *vesicatoria*, *rpoN* is clearly not involved in *hrp* gene expression and pathogenicity (T. Horns and U. Bonas, manuscript in preparation).

Recently, Miller et al. (1993) have reported the identification of two new loci, *hrpQ* and *hrpT*, from *P. s.* pv. *phaseolicola* that affect activation of *hrpRS* in *trans*. However, since *hrpRS* is strongly induced in plants while both *hrpQ* and *hrpT* are constitutively expressed, there must be more factors involved in *hrp* gene regulation. Strains carrying mutations in either *hrpQ* or *hrpT* are amino acid auxotrophs (methionine and tryptophan). *hrpQ* and *hrpT* are probably involved in methionine and tryptophan biosynthesis, respectively (Miller et al. 1993). As stated above, such mutants would normally have been eliminated from the *hrp* mutant analysis.

6.3 Conserved Sequence Boxes in *Pseudomonas syringae*

A conserved sequence, the so-called harp box (TG(A/C)AANC, Fellay et al. 1991), upstream of four *hrp* loci in *P. s.* pv. *phaseolicola*, was suggested to be involved in positive regulation of expression. Similar motifs were described for the promoter regions of several *P. syringae* avirulence genes, the expression of which is dependent on *hrpRS* and on *rpoN* (Huynh et al. 1989; Salmeron and Staskawicz 1993; Innes et al. 1993; Shen and Keen 1993). These studies led to a revised 'harp' box sequence (GGAACCNA). Its significance in protein binding has not been shown but *avrD* promoter constructs lacking the harp box are no longer inducible (Shen and Keen 1993). A harp box-related motif was also found upstream of transcription unit 3 in *P. solanacearum* (Gough et al. 1993).

There is no harp box sequence in *Xanthomonas hrp* gene promoters. Another sequence motif that occurs in the promoter region of *hrp* loci in *X. c.* pv. *vesicatoria* was recently identified. This "PIP" (plant-inducible promoter) box has the sequence TTCGC-N15-TTCGC and occurs upstream of the –35 consensus sequence in four out of six *hrp* promoters (S. Fenselau and U. Bonas, unpublished). Experiments are underway to test whether this is a protein binding motif.

6.4 *Xanthomonas campestris*

Expression of *hrp* genes in *X. c.* pv. *campestris* was determined after growth in vitro and found to be induced in a minimal medium with sucrose and/or fructose as carbon source. No expression occurred in complex media or with high concentrations of organic nitrogen (Arlat et al. 1991). In *X. c.* pv. *vesicatoria*, expression of the six *hrp* loci is induced in the plant but cannot be efficiently induced in the synthetic media tested so far. However, culture filtrates of sterile tomato cell suspension cultures (called TCM) induced expression of the six *hrp* loci in *X. c.* pv. *vesicatoria* whereas the basal Murashige-Skoog culture medium did not. The inducing factor(s) could only partially be purified from TCM and was found to be smaller than 1000 dalton, heat-stable, organic, and hydrophilic (Schulte and Bonas 1992a). De novo transcription of all *hrp* loci occurs rapidly within 1 h after transfer of the bacteria into TCM (S. Fenselau and U. Bonas, unpublished). A minimal medium was designed which would not suppress *hrp* gene induction. This medium, called XVM1, induces the *hrpF* locus (Fig. 1A) to high levels and differs from the other media described above, in particular by its low concentration in phosphate. Both sucrose and methionine are needed for efficient induction. It is not known whether a plant factor is necessary for activation of the other *hrp* loci, or if the XVM1 medium still lacks components or contains them in suppressing amounts (Schulte and Bonas 1992b).

6.5 *Erwinia* and *Pseudomonas solanacearum*

The *hrp* genes of *Erwinia amylovora* are rapidly induced in the non-host, tobacco, and more slowly in the host, pear. Several loci were induced in minimal medium with mannitol as a carbon source. Induction was suppressed by high concentrations of nitrogen and by glucose and was slightly temperature dependent (Wei et al. 1992b).

In *P. solanacearum*, the *hrp* cluster was also induced in host and in non-host plants, as well as in minimal medium. The best carbon sources for induction of four of the six transcription units were pyruvate and glutamate while, as in other bacteria, casamino acids suppressed induction (Arlat et al. 1992). The two rightmost *hrp* transcription units (5 and 6; Fig. 1B) are constitutively expressed but can be induced under certain conditions (Genin et al. 1992).

The only other gene reported to regulate *hrp* gene expression is *hrpB* from *P. solanacearum*. The gene is part of the *hrp* cluster and appears to be a member of the AraC family of positive regulatory proteins. Interestingly, *hrpB* is related to *virF* of *Yersinia* (Cornelis et al. 1989; Genin et al. 1992). The *hrpB* gene positively regulates four of the six *hrp* loci, as well as the *popA* locus, located outside of the *hrp* cluster which encodes a protein secreted in a Hrp-dependent way (see above; Arlat et al. 1994). Whether the HrpB protein binds directly to *hrp* promoters is not yet known.

At this time one can only speculate whether the regulatory systems for *hrp* gene expression employed by *P. solanacearum* and *P. syringae* are really different

or whether there is a global regulatory network thus allowing the fine tuning of gene expression in response to environmental cues. In conclusion, most *hrp* loci from different bacteria are inducible in a particular minimal medium. At this time it cannot be ruled out that stimulation of *hrp* gene expression involves specific plant factors as was described for the virulence genes of *Agrobacterium* (WINANS 1992). Since the composition of the nutrients available to the pathogen in the plant is not known one can only speculate that the conditions described above reflect the situation in the plant. It is noteworthy that the in vitro culture will only mimic the dynamic nutritional situation that bacteria experience in their interaction with a plant for a short time. In mammalian bacterial pathogens, the expression of genes involved in virulence is also regulated in response to environmental cues rather than to specific host molecules. This subject has been reviewed recently (MEKALANOS 1992 and in accompanying chapters), and I will only mention some important factors. In *Yersinia*, the *vir* and *lcr* genes are regulated by low calcium (low calcium response genes; STRALEY et al. 1993) and by temperature (CORNELIS et al. 1989; see chapter by CORNELIS). A calcium effect has not been described for any plant bacterium. In our laboratory no effect of calcium on *hrpF* gene expression in XVM1 was observed (Schulte and U. Bonas, unpublished). Expression of *invA* of *S. typhimurium* of the *mxi* and *ipa* genes of *Shigella* is affected by osmolarity and the later genes also by temperature (GALÁN and CURTISS 1990; HALE 1991).

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Th Enigmatic Avirulence Genes of Phytopathogenic Bacteria

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1 Action at the "Pathogenic Cusp"

The previous chapters have discussed how phytopathogenic bacteria can sense and respond to conditions present in a variety of microenvironments: soil, water, plant cell surfaces, and intracellular spaces. The switch from epiphyte to pathogen is apparently accompanied by fundamental reprogramming of gene activity and attendant function, as evidenced by induction of *hrp* genes and subsequent production of various virulence and pathogenicity factors, some of which are host-specific, some not. This reprogramming switch between epiphytic and pathogenic growth strategies, "the pathogenic cusp" (Dangl 1994), is the point at which not only the potential pathogen but also the host first sense and respond to each other. A successful plant defense response should be based on surveillance and interdiction before the pathogen has a chance to establish production of the armory of factors which determine successful colonization of that host. It is incumbent on each potential plant host, then, to evolve mechanisms to recognize some factor, preferably one produced at this pathogenic cusp, and to base resistance strategies on early recognition. Thus, an evolutionary tug-of-war is

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EXHIBIT 4

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The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae*, *glycinea*, and *tomato* Are Encoded by an Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive Response in Tomato but not Soybean

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The *Pseudomonas syringae* pathovars are composed of h st-specific plant pathogens that characteristically elicit the defense-associated hypersensitive response (HR) in n nhost plants. *P. s.* pv. *syringae* 61 secretes an HR elicit r, harpin$_{Pss}$ (HrpZ$_{Pss}$), in a *hrp*-dependent manner. An internal fragment of the *P. s.* pv. *syringae* 61 *hrpZ* gene was used to clone the *hrpZ* locus from *P. s.* pv. *glycinea* race 4 (bacterial blight of soybean) and *P. s.* pv. *tomato* DC3000 (bacterial speck of tomato). DNA sequence analysis revealed that *hrpZ* is the second ORF in a polycistronic operon. The amino acid sequence identities of HrpZ$_{Pss}$/HrpZ$_{Psg}$ and HrpZ$_{Pss}$/HrpZ$_{Pst}$ were 79 and 63%, respectively. Although none of the HrpZ proteins showed significant overall sequence similarity with other known proteins, HrpZ$_{Pst}$ contained a 24-amino acid sequence that is h mologous with a region of the PopA1 elicitor protein of the tomato pathogen, *Pseudomonas solanacearum* GMI1000. *hrpA*, the upstream ORF, was highly divergent: The amino acid sequence identities of HrpA$_{Pss}$/HrpA$_{Psg}$ and HrpA$_{Pss}$/HrpA$_{Pst}$ were 91 and 28%, respectively, and n HrpA sequence showed similarity to known proteins. In contrast, the predicted products of the downstream ORFs in *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, *hrpB*, *hrpC*, *hrpD*, and *hrpE* showed varying levels of similarity to those of *yscI*, *yscJ*, *yscK*, and *yscL*. These are colinearly arranged genes in the *virC* locus of *Yersinia* spp., which are involved in the secretion of the Yop virulence proteins via the type III pathway. The similarity of the Ysc proteins was generally stronger in comparisons with the *P. s.* pv. *tomato* Hrp proteins. The HrpZ proteins were purified by heat denaturation of contaminating proteins followed by ammonium sulfate fractionation, hydrophobic chromatography, and gel electrophoresis. All three HrpZ proteins elicited the HR in tomato, whereas none of them elicited significant necrosis in s ybean. The results indicate that HrpZ is enc ded in an operon containing some of the genes inv lved in its own secreti n and suggest that HrpZ structure does not directly determine bacterial host range.

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Phytopathogenic strains of *Pseudomonas syringae* cause two patterns of necrosis when the bacteria invade a plant. On a susceptible ("compatible") host, a necrotic lesion often develops over a period of days, with necrosis spreading as the bacteria multiply and the plant becomes diseased. On a resistant or nonhost plant, a localized cellular necrosis is induced within 24 to 48 h, and bacterial multiplication is inhibited. This was first reported by Klement (1963; Klement et al. 1964), who observed that when high concentrations of pathogenic bacteria are infiltrated into an incompatible plant they elicit a visible necrosis which is limited to the infiltrated area. This reaction, called the hypersensitive response (HR), involves localized cell death and production of anti-microbial compounds at the site of pathogen invasion (Bonas 1994).The ability of *P. syringae* and other nontumorigenic, gram-negative, bacterial pathogens to elicit the HR is governed by *hrp* genes. Typical Hrp$^-$ mutants are pleiotropically defective in planta: They do not elicit the HR in nonhosts and they fail to multiply and cause disease in host plants (Lindgren et al. 1986). Clusters of *hrp* genes have been identified in many gram-negative phytopathogenic bacteria (Bonas 1994). A 25-kb *hrp* cluster from *P. s.* pv. *syringae* 61 is sufficient to confer the tobacco HR phenotype, but not the pathogenic phenotype on nonpathogenic bacteria (Huang et al. 1988). *hrp* genes have also been cloned and characterized extensively from *P. s.* pv. *phaseolicola* NPS3121, *P. solanacearum* GM1000, *Xanthomonas campestris* pv. *vesicatoria* 75-3, and *Erwinia amylovora* Ea321 (Lindgren et al. 1986; Boucher et al. 1987; Beer et al. 1991; Bonas et al. 1991). Certain *hrp* genes are widely conserved among these pathogens, and several encode components of a protein secretion pathway that is similar to the type III pathway used by *Yersinia*, *Shigella*, and *Salmonella* spp. to secrete extracellular proteins involved in animal pathogenesis (Van Gijsegem et al. 1993). One activity of the *hrp*-encoded secretion pathway in phytopathogenic bacteria is the secretion of proteinaceous elicitors of the HR, which are also encoded by *hrp* genes.

The first *hrp*-encoded elicitor characterized was harpin$_{Ea}$ from *E. amylovora* (Wei et al. 1992). Similar elicitors have since been isolated from other bacteria, including *P. s.* pv. *syringae* 61, *P. solanacearum* GMI1000, and *E. chrysanthemi*

EC16 (He et al. 1993; Arlat et al. 1994; Bauer et al. 1994). Proteins in this family of elicitors share several general characteristics. They are glycine rich, heat-stable, lack cysteine, and appear highly susceptible to proteolysis. They lack an N-terminal signal peptide, but they are secreted to the bacterial milieu. Their expression and secretion is dependent on *hrp* genes. The biological role of these proteins in pathogenesis has not yet been determined, but the purified proteins can induce an HR on a nonhost plant such as tobacco. However, there are significant differences in the organization of the elicitor operons and the activity of the elicitors, which suggests that the *Erwinia* harpins, the *P. syringae hrpZ* product and the *P. solanacearum popA* product may represent three distinct classes of elicitors. In this work we will refer to the *P. s.* pv. *syringae* elicitor as $HrpZ_{Pss}$ rather than $harpin_{Pss}$ (He et al. 1993). This distinction is supported by the weak similarity of the amino acid sequences of the four proteins, with the only exception being the C-terminal halves of the *Erwinia* harpins (Bauer et al. 1994).

The location of known elicitor genes in reference to the *hrp* cluster varies in *P. s.* pv. *syringae*, *P. solanacearum*, and *E. amylovora*. *hrpN* and *hrpZ* are contiguous or within the *hrp* cluster, whereas *popA* lies outside (although near) the *P. solanacearum hrp* cluster (Wei et al. 1992; He et al. 1993; Arlat et al. 1994). There are no genes downstream of the elicitor gene in either the *hrpN* or the *popA* operons, which means that mutations in the elicitor genes do not have a polar effect on the Hrp phenotype, and mutant construction is straightforward. In contrast, mutagenesis and complementation studies of the *hrp* cluster from *P. s.* pv. *syringae* 61 have indicated that *hrpZ* lies upstream of at least one other *hrp* gene within an operon (Huang et al. 1991; Xiao et al. 1992).

In *E. amylovora* and *E. chrysanthemi*, harpins have been demonstrated to be sufficient and necessary to elicit the HR, and mutation of *hrpN* in *E. amylovora* has shown that $harpin_{Ea}$ is required for pathogenesis (Wei et al. 1992). However *hrpN* mutants of *E. chrysanthemi* can establish infections, albeit at a significantly reduced frequency, which suggests that $harpin_{Ech}$ is important but not essential for pathogenesis (Bauer et al. 1995). In contrast, a *popA* mutant of *P. solanacearum* is fully pathogenic on susceptible hosts, indicating that PopA1 is not required for pathogenesis (Arlat et al. 1994).

These elicitors may play a role in controlling the host specificity exhibited by *E. amylovora* and plant pathogenic pseudomonads such as *P. syringae* and *P. solanacearum*. However it is difficult to compare the activity of $HrpZ_{Pss}$ and $harpin_{Ea}$ in host and nonhost plants because legumes and rosaceous plants, the hosts of *P. s.* pv. *syringae* 61 and *E. amylovora* Ea321, respectively, respond poorly to preparations of any of these elicitor proteins (Wei et al. 1992; He et al. 1993). PopA1 from *P. solanacearum* does appear to act in a host-specific manner, inducing an HR on resistant lines of petunia and the nonhost tobacco, but not on susceptible lines of petunia or tomat (Arlat et al. 1994). This phenotype is similar to that of *avr* genes, but PopA1 is distinct from known Avr proteins in eliciting the HR directly on resistant plants. $Harpin_{Ech}$ elicits an HR on some compatible hosts of *E. chrysanthemi*, but in contrast to the other three bacteria *E. chrysanthemi* is a broad-host range pathogen and the activity of $harpin_{Ech}$ may not be representative of elicitor activity in a highly host-specific system (Bauer et al. 1995).

In previous work we cloned and characterized the *hrpZ* gene from *P. s.* pv. *syringae* 61, a weak pathogen of bean, and demonstrated with Southern and immunoblots that other pathovars of *P. syringae* contain homologs of this gene (He et al. 1993). This supported the hypothesis that HrpZ represents a family of elicitors common to all pathogenic strains of *P. syringae*. We report here the isolation of homologs of $HrpZ_{Pss}$ from two other experimentally important pathovars of *P. syringae-P. s.* pv. *tomato* and *P. s.* pv. *glycinea*. Examining HrpZ from these three pathovars enabled us to look within this family of elicitors for variations in sequence and activity which could indicate a role in host range determination. In addition, we characterized the two genes flanking *hrpZ* in *P. s.* pv. *syringae* and *P. s.* pv. *glycinea* and the entire *hrpZ* operon of *P. s.* pv. *tomato*. In conjunction with an accompanying paper (Huang et al. 1995), this completes the sequence of the *P. s.* pv. *syringae* 61 *hrp* genes carried on pHIR11 and provides clues to the function of the genes downstream of *hrpZ*. A preliminary account of portions of this work has been published (Collmer et al. 1994).

RESULTS

Cloning *hrpZ* from *P. s.* pv. *tomato* and *P. s.* pv. *glycinea*.

We previously used Southern hybridization to demonstrate that both *P. s.* pv. *glycinea* race 4 and *P. s.* pv *tomato* DC3000 contain sequences homologous to a 0.75 kb *Bst*XI internal fragment of *hrpZ* from *P. s.* pv. *syringae* (He et al. 1993). The same probe was used to screen genomic libraries of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*. The libraries were constructed in *E. coli* DH5α by inserting 8- to 12-kb fragments from partial *Sau*3AI digests of genomic DNA into the *Bam*HI site of pUCP19. The screen identified two plasmids with inserts of approximately 10 kb: pCPP2201 (*P. s.* pv. *tomato*) and pCPP2200 (*P. s.* pv. *glycinea*). The same *Bst*XI fragment was used to probe a Southern blot of pCPP2201 and pCPP2200 digested with *Bam*HI, *Eco*RI, and *Pst*I. The probe identified two *Pst*I fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200 respectively (Fig. 1). The two *Pst*I fragments were cloned into the *Pst*I site of pBluescript II SK(–) (Stratagene, La Jolla, CA) in *E. coli* DH5α to create the plasmids pCPP2202 to pCPP2205, with the inserts in both orientations with respect to the *lac* promoter. Cell lysates of *E. coli* DH5α containing pCPP2203 ($hrpZ_{Pss}$ in the vector promoter orientation) and pCPP2202 ($hrpZ_{Psg}$ in the vector promoter orientation) induced an HR on tobacco, but those from cells containing pCPP2205 ($hrpZ_{Pss}$ in the opposite orientation of the vector promoter) and pCPP2204 ($hrpZ_{Psg}$ in the opposite orientation of the vector promoter) did not. HR activity was retained after incubating the lysate for 10 min at 100°C and removing denatured proteins by centrifugation. Insensitivity to heat treatment is a characteristic feature of previously isolated HR elicitors. Proteins in the lysates were separated on an SDS-polyacrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibodies raised against purified $HrpZ_{Pss}$. Cross-r acting proteins of a similar size to $HrpZ_{Pss}$ were observed and provisionally named $HrpZ_{Psg}$ and $HrpZ_{Pss}$ (Fig. 2, lanes 2 and 4).

The intensity of the $HrpZ_{Psg}$ and $HrpZ_{Pss}$ bands was quite low in comparison t the band for $HrpZ_{Pss}$ expressed from pSYH10 in *E. coli* DH5α (Fig. 2, lane 1). This implied either

that expression was low due to the distance of the cloned gene from the *lac* promoter or that $HrpZ_{Pss}$ and $HrpZ_{Psi}$ did not hybridize strongly to the antibodies. A band corresponding to $HrpZ_{Pss}$ from pSYH10 could be clearly seen on a Coomassie-stained gel, but the bands for $HrpZ_{Pss}$ and $HrpZ_{Psi}$ were indistinct, which implies that low expression was a primary reason for the low signal. In an attempt to improve the level of expression of $HrpZ_{Pss}$ and $HrpZ_{Psi}$ we subcloned *Eco*RI-*Bam*HI fragments containing the inserts from pCPP2202 and pCPP2203 behind the T7 promoter of pET21(+) in *E. coli* BL21(DE3) to create the plasmids pCPP2206 and pCPP2207. The T7 promoter enabled a moderate improv ment in protein expression (Fig. 2, lanes 3 and 5).

A common arrangement of ORFs in the *hrpZ* operons of *P. s.* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato* revealed by DNA sequence analysis.

Previously, we determined the complete nucleotide sequence of *hrpZ* from *P. s.* pv. *syringae* by sequencing a 1.4-kb subclone of pHIR11 (a cosmid containing the entire *hrp* cluster from *P. s.* pv. *syringae*) (He et al. 1993). In addition, analysis of the complementation groups and transcriptional

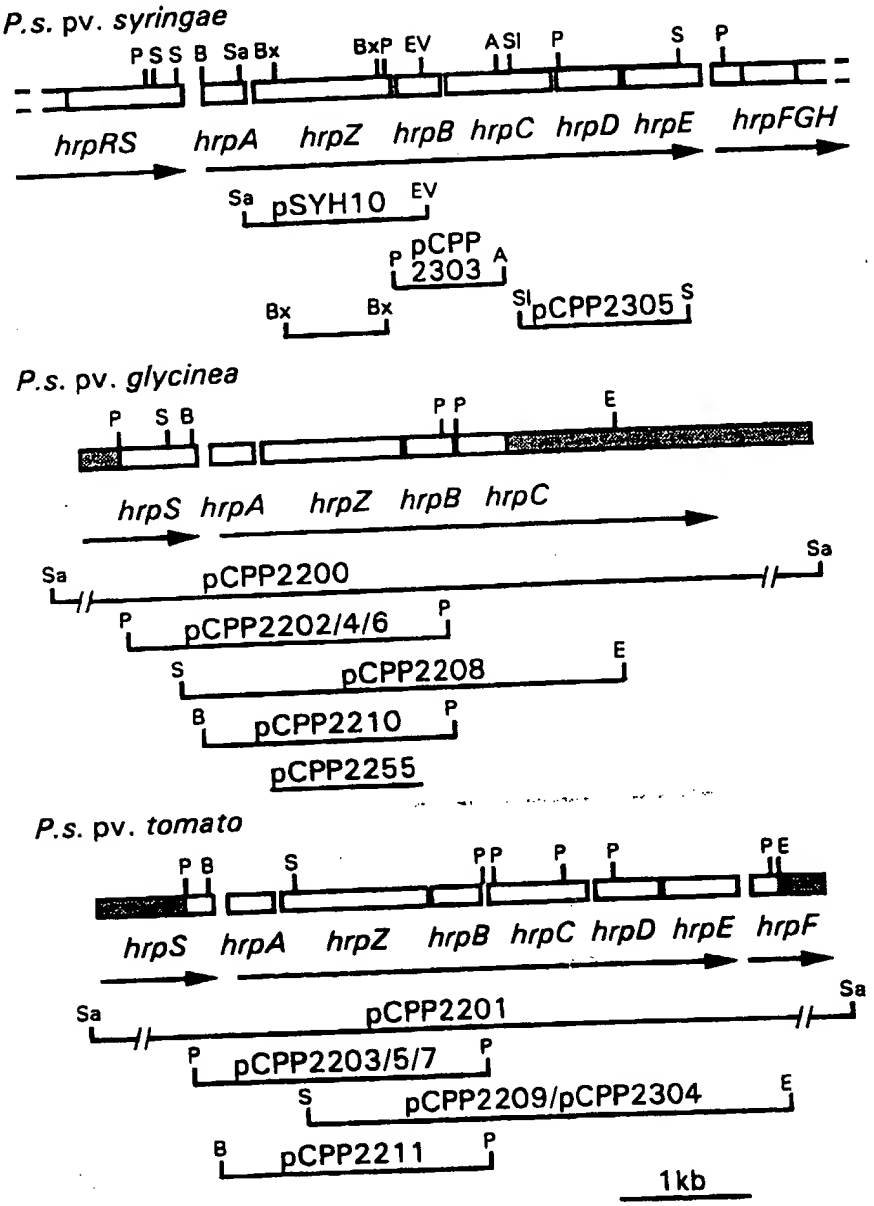

Fig. 1. Physical maps of the *hrpZ* regions from *Pseudomonas syringae* pv. *syringae* 61, *P. s.* pv. *glycinea* race 4, and *P. s.* pv. *tomato* DC3000 and clones used in this study. Open boxes represent sequenced ORFs; filled boxes represent unsequenced DNA. Direction of transcription is indicated by the arrows. Key restriction sites within the sequenced regions are indicated, along with the subclones used in this study. The 0.75-kb *Bst*XI fragment from $hrpZ_{Psi}$ used as a probe for *hrpZ* genes in other pathovars is also shown. Restriction endonuclease abbreviations: A, *Age*I*; B, *Bgl*II; Bx, *Bst*XI*; E, *Eco*RI; EV, *Eco*RV*; P, *Pst*I; S, *Sac*I; Sa, *Sau*3A*; SI, *Sal*I*. * Not all sites are shown.

units of pHIR11 using Tn*phoA* and Tn5-*gusA*1 mutagenesis (Huang et al. 1991; Xiao et al. 1992) suggested that *hrpZ* lay within an operon, upstream of at least one other *hrp* gene. Further subclones of pHIR11 were used to determine the sequence of the entire $hrpZ_{Pss}$ operon (this study, Huang et al. 1995). We also determined the sequence of (i) the 2.2- and 2.4-kb *Pst*I subclones from pCPP2201 ($hrpZ_{Pss}^{+}$) and pCPP2200 ($hrpZ_{Psg}^{+}$), (ii) an overlapping 3.7-kb *Sac*I-*Eco*RI subclone from pCPP2201 (designated pCPP2209), and (iii) part of an overlapping 3.6-kb subclone from pCPP2200 (designated pCPP2208), as shown in Figure 1. This yielded the sequence of the entire *P. s.* pv. *tomato hrpZ* operon and the first half of the *P. s.* pv. *glycinea* operon. The sequenced region of *P. s.* pv. *syringae* and *P. s.* pv. *tomato* extends from *hrpS* (Xiao et al. 1994), through the *hrpZ* operon to the beginning of the *hrpH* operon (Huang et al. 1992), demonstrating that the organization of this region of the *hrp* cluster is conserved in both pathovars.

Codon preference analysis of the DNA sequence, using *P. s.* pv. *syringae* codon usage data, predicted that *hrpZ* was the second of six ORFs, all oriented in the same direction, an arrangement conserved in *P. s.* pv. *tomato* and at least the first four ORFs of *P. s.* pv. *glycinea*. The sequence of the noncoding DNA is shown in Figure 3. Five of the six ORFs have clear potential ribosome binding sites. The fifth ORF has a putative ribosome binding site in *P. s.* pv. *syringae*, but the site in *P. s.* pv. *tomato* is less clear, the initiation codon shown being selected by alignment with the ORF in *P. s.* pv. *syringae*. In the absence of recognizable terminator elements downstream of the first five ORFs it seems likely that the six ORFs represent a single operon, transcribed from upstream of the first ORF. The five predicted ORFs were provisionally named *hrpA* through *hrpE*, as shown in Figures 1 and 3.

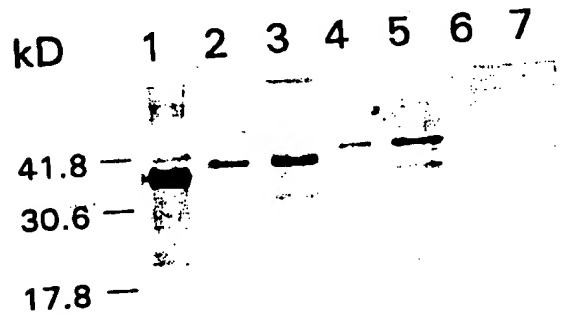

Fig. 2. Immunoblot showing expression of cloned *hrpZ* in *E. coli*. Cultures were grown in LM to an OD_{600} of 0.8 to 1.0 at 30°C, collected by centrifugation and resuspended in 5 mM MES, pH 5.5. For lanes 3, 5 and 7, and 4, T7 expression was induced with 1 mM IPTG when the cells reached an OD_{600} of 0.6, 3 h prior to collection. The cells were disrupted by sonication, and the crude lysate was partially purified by removal of the insoluble fraction after incubating the samples at 100°C for 10 min. SDS-loading buffer was added and the samples were incubated at 100°C for 2 min. The proteins were resolved by SDS-polyacrylamide gel electrophoresis. Following electrophoresis the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), probed with anti-$HrpZ_{Pss}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α (pSYH10) ($HrpZ_{Pss}$); 2, *E. coli* DH5α (pCPP2202)($HrpZ_{Psg}$) 3, *E. coli* BL21(DE3) (pCPP2206)($HrpZ_{Psg}$); 4, *E. coli* DH5α (pCPP2203)($HrpZ_{Pss}$); 5, *E. coli* BL21(DE3) (pCPP2207)($HrpZ_{Pss}$); 6, *E. coli* DH5α (pBluescript II); 7, *E. coli* BL21(DE3)(pET21+).

A *hrp/avr* promoter consensus sequence lies upstream of the *hrpZ* perons of the three *P. syringae* pathovars.

The conserved sequence GGAACC—16bp—CCACNNA lies 50 bp upstream of the initiation codon of *hrpA* in all three pathovars (Fig. 3). This motif has been identified in the promoter regions of many *avr* and *hrp* genes (Innes et al. 1993; Shen and Keen 1993), and appears to be involved in positive regulation by HrpL, a putative alternative sigma factor which is itself positively regulated by HrpR and HrpS (Xiao and Hutcheson 1994). HrpL is a member of a family of alternative sigma factors, many of which are involved in secretion of extracellular factors in response to environmental stimuli (Lonetto et al. 1992). The presence of this promoter motif further supports the suggestion that the six ORFs form a single transcriptional unit which is regulated in a *hrp*-dependent manner. This motif can also be found beyond *hrpE*, upstream of *hrpFGH* in *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, as indicated at the bottom of Figure 3, suggesting that the latter three ORFs form an independent *hrp*-regulated transcriptional unit in these two pathovars.

Comparison of the HrpZ proteins of the three *P. syringae* pathovars.

The predicted amino acid sequences for HrpZ from each of the three pathovars are aligned in Figure 4. Although the proteins migrate slightly anomalously on an SDS polyacrylamide gel, the relative sizes of the estimated molecular weights correspond to the predicted values, with $HrpZ_{Pss}$ being the largest of the three proteins (36.5 kDa), followed by $HrpZ_{Psg}$ (35.3 kDa) and $HrpZ_{Psa}$ (34.7 kDa). Amino-terminal sequencing of the first 10 to 15 residues of purified $HrpZ_{Psg}$ and $HrpZ_{Pss}$ confirmed the predicted initiation codons of both proteins, which aligned with the start codon of $HrpZ_{Pss}$ as shown in Figures 3 and 4. The proteins expressed in *E. coli* appear to be the same size as those recovered from the supernatants of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*, indicating that the cloned gene is intact and that there are no large post-translational modifications or deletions of HrpZ taking place in *P. syringae* but not in *E. coli*.

The amino acid sequence of $HrpZ_{Psg}$ is quite highly conserved with respect to $HrpZ_{Pss}$, having 87% similarity and 79% identity. $HrpZ_{Psa}$ is less conserved with respect to the two other proteins, with 75% similarity and 63% identity to $HrpZ_{Pss}$. However, the physical features of $HrpZ_{Psg}$ and $HrpZ_{Psa}$ are almost identical to those reported for $HrpZ_{Pss}$ (He et al. 1993). All three are glycine-rich proteins lacking cysteine and tyrosine. $HrpZ_{Psa}$ is the most glycine rich, being 15.7% glycine. The proteins lack the hydrophobic signal sequence used to target proteins for secretion via the Sec export pathway (Pugsley 1989). Analysis of the amino acid sequence fails to identify any obviously significant secondary structure, which is consistent with their sensitivity to proteases, and supports the suggestion that they adopt a fairly open structure in aqueous solution.

In our previous analysis of $HrpZ_{Pss}$ (He et al. 1993), we noted the presence of two sets of short, direct repeats. Only one of these repeats, GGGLGTP, is conserved in the three proteins, with the substitution of a serine f r threonine in the first repeat of both $HrpZ_{Psg}$ and $HrpZ_{Psa}$. The significance of these repeats, if any, is unknown. A database search with each of the three proteins using the BLAST alg rithm (Altschul et

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syringae   TTTTTTGCAG AAGATCTGGA ACCGATTCGC GGACACATGC CACCTAGCTG
glycinea   TTTTTTGCA. GAGCGCTGGA ACCGATTTAA GGGTCGTTAC CACTA.TCTG
  tomato   TTTTTTGCAA AGACGCTGGA ACCGTATCGC AGGCTGCTGC CACTAGTGAG

syringae   TACCAAGCAA TTACGCTGGT ACAGACGAAG GGGTATGACG TTATG-----
glycinea   TACCAAGCAA TTACGCTGGT ACAGACCAAG GGGTATCACG TTATG-----
  tomato   TACCAAGCAA TCACGCTGGT AAATCTTAAG GGGCATCAAA TCATG-----

syringae   ---------- --321bp--- ---------T GATTTCTTG. ACGCCCCTTC
glycinea   ---hrpA--- --321bp--- ---------T GATTTCTTGA ATGCCCCCAT
  tomato   ---------- --336bp--- ---------T AATTATTTCT GATTGCCCCC
                                                        ======
syringae   ATACCTGAGG GGGCTGCTAC TTTTAGGAGG TTGTG..ATG ----------
glycinea   CACACAGAGG GGGCTGCTAC TTTGAGGAGG TTGTG..ATG ----------
  tomato   TCATCAGAGG GGGCCGCTAC CTTGGGATGG GCGTTTTATG ----------
           =>    <=== ====
syringae   ---------- --1020bp-- ---------- ---------- ----------
glycinea   ---hrpZ--- --1032bp-- ---------- ---------- ----------
  tomato   ---------- --1107bp-- ---------- ---------- ----------

syringae   ---------- ---------- ---------- ---------- ----------
glycinea   ---------- ---------- ---------- ---------- ----------
  tomato   ---------- ---------- ---------- ---------- ----------

syringae   TGACCGACAA CCGCCTGACG GAGAACTCAC GTG------- ----------
glycinea   TGACTGATAC CCGCCTGACG GAGAACTCAC GTG------- ---hrpB---
  tomato   TGACTGACAG CCGCCTGACG GAGAACCAGT GTG------- ----------

syringae   --369bp--- ---------- TAGAGGTTTC CGTG------ ----------
glycinea   --369bp--- ---------- TAGAGGTTCT CGTG------ ----------
  tomato   --369bp--- ---------- TAGAGGTTTC CGTG------ ----------

syringae   ---------- --801bp--- ---------- ---------- ----------
glycinea   ---hrpC--- incomplete
  tomato   ---------- --801bp--- ---------- ---------- ----------

syringae   ---------- -----TGATG GACCTGACCG CCGAGGACTA TTGGACTCAG
  tomato   ---------- ----ATGATG AGCCTTTCTG CCGAGGATCA CTGGATTCAC

syringae   TGGTGGTGCA ATCCCTGGCC ATGGGCGCAT CCGGGCTGGC AAAGCCGGTT
  tomato   TGGTGGTGCA ACCCCTGGCA GTGGGCACAT TCGGAGTGGC ATGACCGATT

syringae   CGCCGAGCGC TGCGGACTGA CCGTCAGCGA ATGTGAAGCC CTTATG----
  tomato   CGCCAACGCT CGTGGGTTAT CGGTCAGTGA CTGCGATGCG CTCATG----

syringae   ---------- ---hrpD--- --396bp--- ---------- ----------
  tomato   ---------- ---------- --396bp--- ---------- ----------

syringae   --TGAGTAT. .CCGCTCCTC TCTGCACCAG GAATTCTCCC ATG-------
  tomato   --TGAATCCG AACCAGCTTC TCTGCATCAG GAATACGCCC ATG-------

syringae   ---hrpE--- --576bp--- ---------- ---------- -------TGA
  tomato   ---------- --576bp--- ---------- ---------- -------TGA

syringae   AACAGACT.. .........C TTGCGGCGAA AATGGAACCG CTCCACCTGT
  tomato   TACACACTCT CTGCACTCAC TTGATCGCAT GATGGAACCG CTCGGCGGGT

syringae   TTGCTCCACT CAAGGTTTGA ACCTTTCTGC TGGAGTATCA GGACATG
  tomato   TTGCTCCACT CAAGGTTTGA ACCCTTCTGC TGGAGCACCA GGACATG
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Fig. 3. Nucleotide sequences of the noncoding regions of the *hrpZ* operon from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato*. The sequences flanking the six ORFs of the *hrpZ* operon were aligned using the PILEUP algorithm (Genetics Computer Group). For *P. s.* pv. *syringae* and *P. s.* pv. *tomato* the sequence extends from immediately downstream of *hrpS* to the end of the operon. For *P. s.* pv. *glycinea* the sequenced region terminates at the beginning of *hrpC*. The proposed initiation and termination codons are highlighted for each ORF. The *hrp/avr* consensus sequences upstream of *hrpA* amd *hrpF* are marked by double lines, with the conserved nucleotides in bold and the putative ribosome binding sites for each ORF underlined. A short inverted repeat upstream of *hrpZ* is also indicated with dashed arrows.

al. 1990) did not find significant homology to any other bacterial proteins, with the exception of a single, glycine rich region found only in $HrpZ_{Pss}$ (Fig. 4). This stretch of 24 amino acids has homology at both the nucleotide and amino acid level to a region of the host-specific elicitor PopA1 from *P. solanacearum*, as shown at the bottom of Figure 4. There is no overall similarity of the amino acid and nucleotide sequences of HrpZ to the HR elicitors characterized from *E. amylovora*, *E. chrysanthemi*, and *P. solanacearum* except to a degree accounted for by their similar composition.

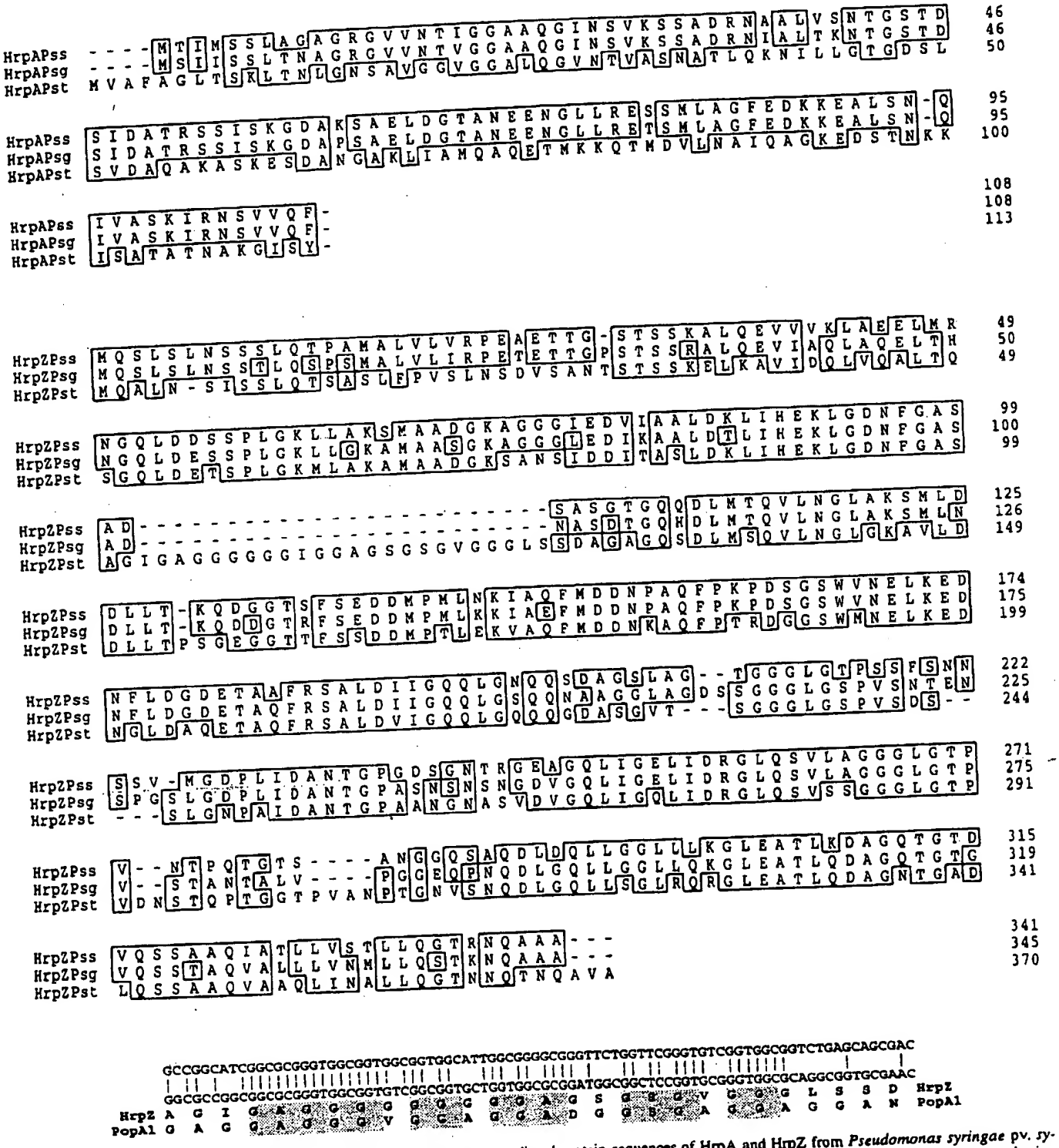

Fig. 4. Alignment of the protein sequences of HrpA and HrpZ. The predicted protein sequences of HrpA and HrpZ from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato* were aligned using the PILEUP algorithm (Genetics Computer Group). The alignment of a unique glycine rich region of $HrpZ_{Pss}$ with a homologous region of PopA1 from *P. solanacearum* is also shown.

The predicted HrpA protein of *P. s.* pv. *tomato* differs substantially from that of *P. s.* pv. *syringae* and *P. s.* pv. *glycinea*.

The first ORF of the *hrpZ* operon starts 50 bp downstream of the conserved *hrp/avr* promoter motif, as shown in Figure 3. The predicted product is a small (11 kDa), hydrophilic protein with a hydrophobic N-terminus. An alignment of the amino acid sequences from all three pathovars is shown in Figure 4. Although the predicted sequences of HrpA from *P. s.* pv. *syringae* and *P. s.* pv. *glycinea* are highly conserved, with 92% similarity and 91% identity to each other, HrpA from *P. s.* pv. *tomato* is quite divergent, having only 42% similarity and 28% identity to HrpA from *P. s.* pv. *syringae* The presence of a ribosome binding site and the highly conserved character of HrpA in two of the three pathovars supports the hypothesis that HrpA is translated. T7 polymerase-dependent expression of *hrpA* (described below) provides further evidence for production of a HrpA protein. Cell lysates of *E. coli* expressing only HrpA did not elicit the HR on tobacco (data not shown), which suggests that it does not contribute directly to the HR. The role of HrpA in the bacterium is unknown, and it shows no significant homology to any previously characterized proteins.

T7 expression studies.

To confirm the production of proteins corresponding to the two sets of newly cloned *hrpA* and *hrpZ* genes, the *Bgl*II-*Pst*I fragments from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* were subcloned into pET21(+) and the products specifically labelled by T7 promoter/polymerase-dependent expression in *E. coli* BL21(DE3) cells incubated with [^{35}S]-methionine (Studier et al. 1990). Radiolabeled proteins in the cell lysate were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 5). Lysates of cells containing pCPP2211 displayed unique bands which corresponded well with the predicted molecular weight of HrpA (11.5 kDa) and were consistent with the previously observed mobility of $HrpZ_{Pst}$ (Fig. 5, lane 2). Lysates of cells containing pCPP2210 contained bands corresponding to $HrpZ_{Psg}$ (36 kDa) and HrpA (11 kDa)(Fig. 5, lane 3). No HrpB band was visible in the products of pCPP2211 (Fig. 5, lane 2), but this could potentially be attributed to the omission of cysteine, which is not required for HrpA and HrpZ synthesis, from the amino-acids added to the reaction mixture. T7 expression of HrpB was independently confirmed for both *P. s.* pv. *syringae* and *P. s.* pv. *tomato* using a 0.84-kb *Pst*I-*Age*I fragment of pHIR11 and the 3.7-kb *Sac*I-*Eco*RI fragment from pCPP2209, subcloned into LITMUS 28 to construct the plasmids pCPP2303 and pCPP2304. T7 expression in *E. coli* BL21(DE3) cells was performed as outlined above and in Figure 5. In each case a protein of about 13 kDa was observed, which corresponds well with the predicted molecular weight of HrpB from each of the two pathovars (data not shown). In an accompanying study Huang et al. (1995) have confirmed the production of proteins corresponding to HrpC, HrpD, and HrpE from *P. s.* pv. *syringae* 61. The similarities between the three pathovars suggest that the equivalent ORFs in *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* also encode proteins. H wever when we independently confirmed the production of HrpD fr m *P. s.* pv. *syringae* 61 using a 1.3-kb *Sal*I-*Sac*I subclone from pHIR11 cl ned into pT7-6 (pCPP2305) our results suggested the use of an alternative initiation codon t make a larger (21 kDa) HrpD protein (data not shown). In the absenc of a strong ribosome binding site at either of the putative initiation codons, the exact size of HrpD remains uncertain.

The four ORFs downstream of *hrpZ* show varying similarities to *Yersinia* Ysc proteins.

The *hrpC*, *hrpD*, and *hrpE* genes downstream of *hrpZ* in *P. s.* pv. *syringae* 61 have been sequenced and the products identified using T7 polymerase-dependent expression (Huang et al. 1995). Two of the predicted proteins, HrpC and HrpE, were shown to be homologous to the proteins YscJ and YscL, respectively, which are encoded in the *virC* operon of *Yersinia enterocolitica* and are involved in the type III secretion pathway (Michiels et al. 1991). Homologs of YscJ have also been found in the *hrp* clusters of several other phytopathogenic bacteria, including *P. solanacearum* and *X. campestris* (Fenselau et al. 1992; Gough et al. 1992). Additional homologs are *Salmonella typhimurium* FliF and *Rhizobium fredii* NolT (Jones et al. 1989; Meinhardt et al. 1993). The same four downstream ORFs are found in *P. s.* pv. *tomato*, and the partial sequence of the operon from *P. s.* pv. *glycinea* confirms the presence of the first two of these ORFs, *hrpB* and *hrpC*, in this pathovar (Fig. 6).

HrpB is fairly conserved in all three pathovars, as shown by the alignment presented in Figure 6. It encodes a small serine-rich protein of approximately 13 kDa. BLAST searches using HrpB from either *P. s.* pv. *syringae* or *P. s.* pv. *glycinea* identified no significant homologies, but a search using HrpB from *P. s.* pv. *tomato* identified similarity to the *Yersinia* protein, YscI. YscI is 115 amino acids long, thus slightly shorter than HrpB (127 amino acids). *yscI* lies immediately upstream of *yscJ* in the *virC* operon, which suggests that the downstream ORFs of the *hrpZ* operon might be colinear with a region of the *virC* operon.

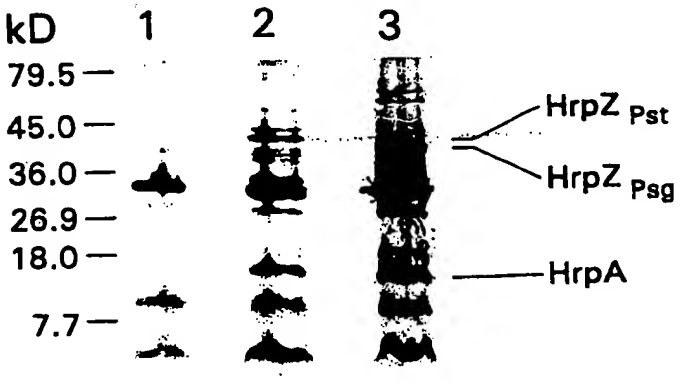

Fig. 5. T7 polymerase-dependent expression and radiolabeling of HrpA and HrpZ. T7 promoter/polymerase expression was carried out using the pET21(+) vector system in *E. coli* BL21(DE3). Cells were grown in LM to an OD_{600} of 0.5, then centrifuged and resuspended in M9 minimal medium supplemented with 0.01% amino acids (lacking methionine and cysteine), glucose and thiamine. Cells were incubated at 30°C for 3 h and then induced with 1 mM IPTG for 10 min, followed by incubation with rifampicin at 300 µg/ml for 30 min. Cells were incubated with 10 µCi [^{35}S]-methionine for 10 min, lysed in SDS-loading buffer, and the proteins were separated by SDS-polyacrylamide electrophoresis and visualized by autoradiography. *E. coli* BL21(DE3) cells carried the following plasmids in lanes: 1, pET21(+); 2, pCPP2211; 3, pCPP2210.

The apparent colinear arrangement of this group of *hrp* and *ysc* genes led us to inspect the *P. s.* pv. *syringae* and *P. s.* pv. *tomato* HrpD proteins for possible similarity to the *Yersinia* spp. YscK proteins. The similarity between the HrpD of *P. s.* pv. *syringae* and *Y. pseudotuberculosis* was the highest, with 28% of the amino acids identical and 57% similar. The HrpD and YscK proteins are of similar overall composition, and they lack any predicted transmembrane segments. However, there is a striking discrepancy between the sizes of the two proteins. HrpD is only 133 amino-acids long, whereas YscK from *Y. pseudotuberculosis* is 209 amino-acids long. From the T7 experiments described above it is important to note that in the absence of a strong ribosome binding site, the precise initiation codon of the *hrpD* ORF is uncertain; it is conceivable that *hrpD* actually initiates immediately downstream of *hrpC*, at the ATG codon which overlaps the st p codon of *hrpC*, which would yield a predicted protein of 176 amino acids for $HrpZ_{Pss}$ or 175 amino acids for $HrpZ_{Psy}$ in an arrangement similar to that of the *yscJ* and *yscK* ORFs in *Yersinia* spp. However, this codon and all other potential initiation codons upstream of the one we have chosen lack ribosome binding sites, and the pattern of codon usage suggests that the intergenic region is not translated.

Although the similarities between HrpB/YscI, HrpD/YscK, and HrpE/YscL are lower than those involving HrpC/YscJ, the similarities of HrpB/YscI and HrpE/YscL are clearly in-

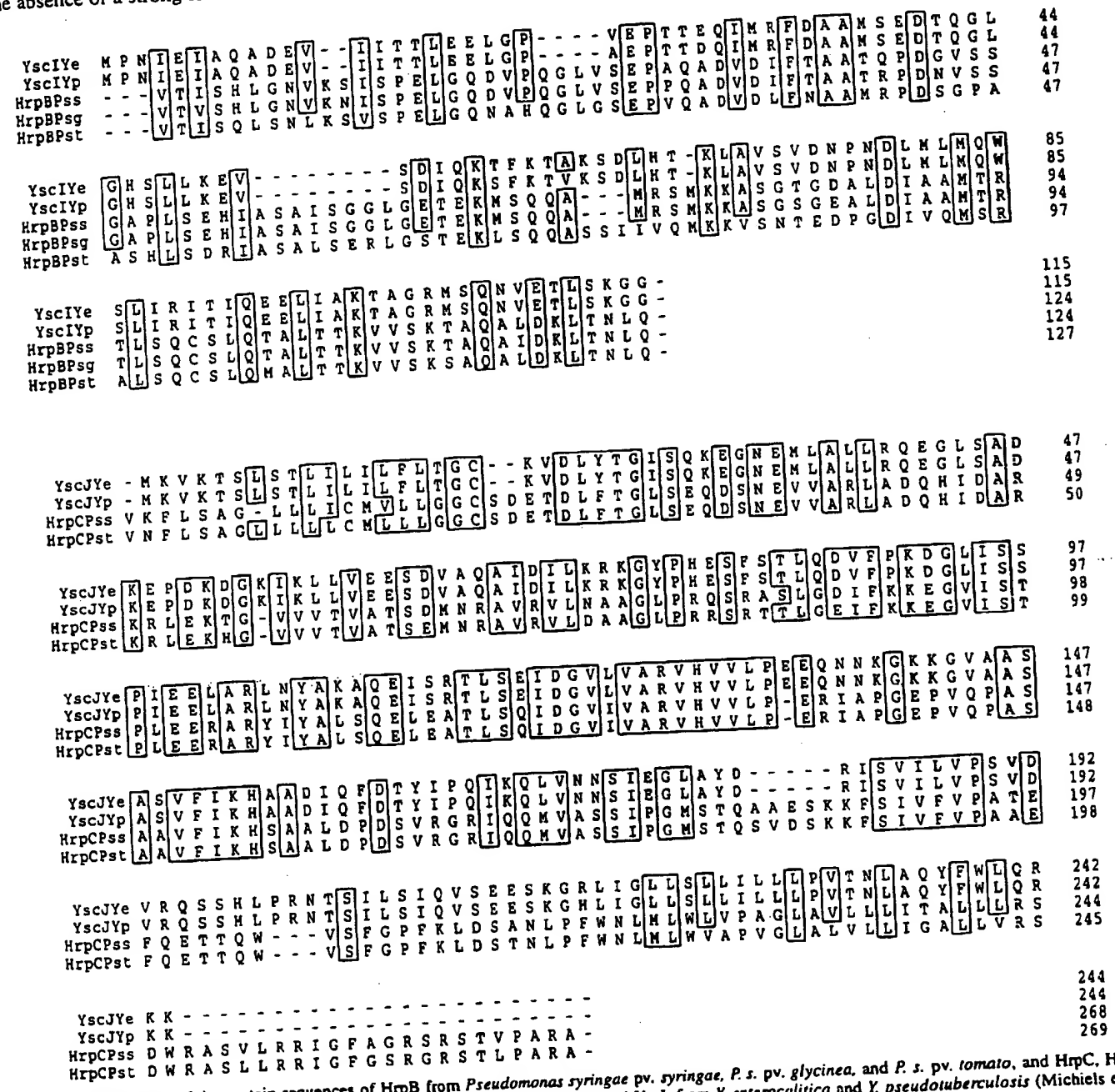

Fig. 6. Alignment of the protein sequences of HrpB from *Pseudomonas syringae* pv. *syringae*, *P. s.* pv. *glycinea*, and *P. s.* pv. *tomato*, and HrpC, HrpD and HrpE from *P. s.* pv. *syringae* and *P. s.* pv. *tomato* with YscI, YscJ, YscK, and YscL from *Y. enterocolitica* and *Y. pseudotuberculosis* (Michiels et al. 1991; Rimpilainen et al. 1992). *(continued on next page)*

dicative of pr bable homology as based on a difference between the scores for the optimized and the average of 100 random Gap alignments being at least 5 times the standard deviation for the randomized alignments (Doolittle 1986). The scores for HrpD/YscK lie at the margin of significance by this measure. However, the varying levels of similarity are consistent with the divergence observed between Hrp proteins from different *P. syringae* pathovars and between Ysc proteins from different *Yersinia* spp. The results for HrpB,C and E lend support to the weak homology of HrpD to YscK and suggest that *hrpB*, *hrpC*, *hrpD*, and *hrpE* are colinear with *yscI*, *yscJ*, *yscK*, and *yscL*.

In a recent report, Van Gijsegem et al. (1995) observe that the *P. solanacearum* GMI1000 *hrp* cluster also encodes homologs of YscJ and YscL but not YscI and YscK. It is possible that with relatively divergent Hrp s quences, similarities with Ysc proteins may be found only after examining the sequences from several plant pathogens. It is interesting to note that there is no ORF following *hrpE* that is homologous to the protein encoded by the final gene of the *virC* operon, YscM. However, the *hrpZ* operon lies immediately upstream of the *hrpH* operon (Fig. 1), and HrpH is a homolog of YscC, a secretion protein which lies upstream of *yscIJKL* within the *virC* operon (Michiels et al. 1991). This suggests that a sig-

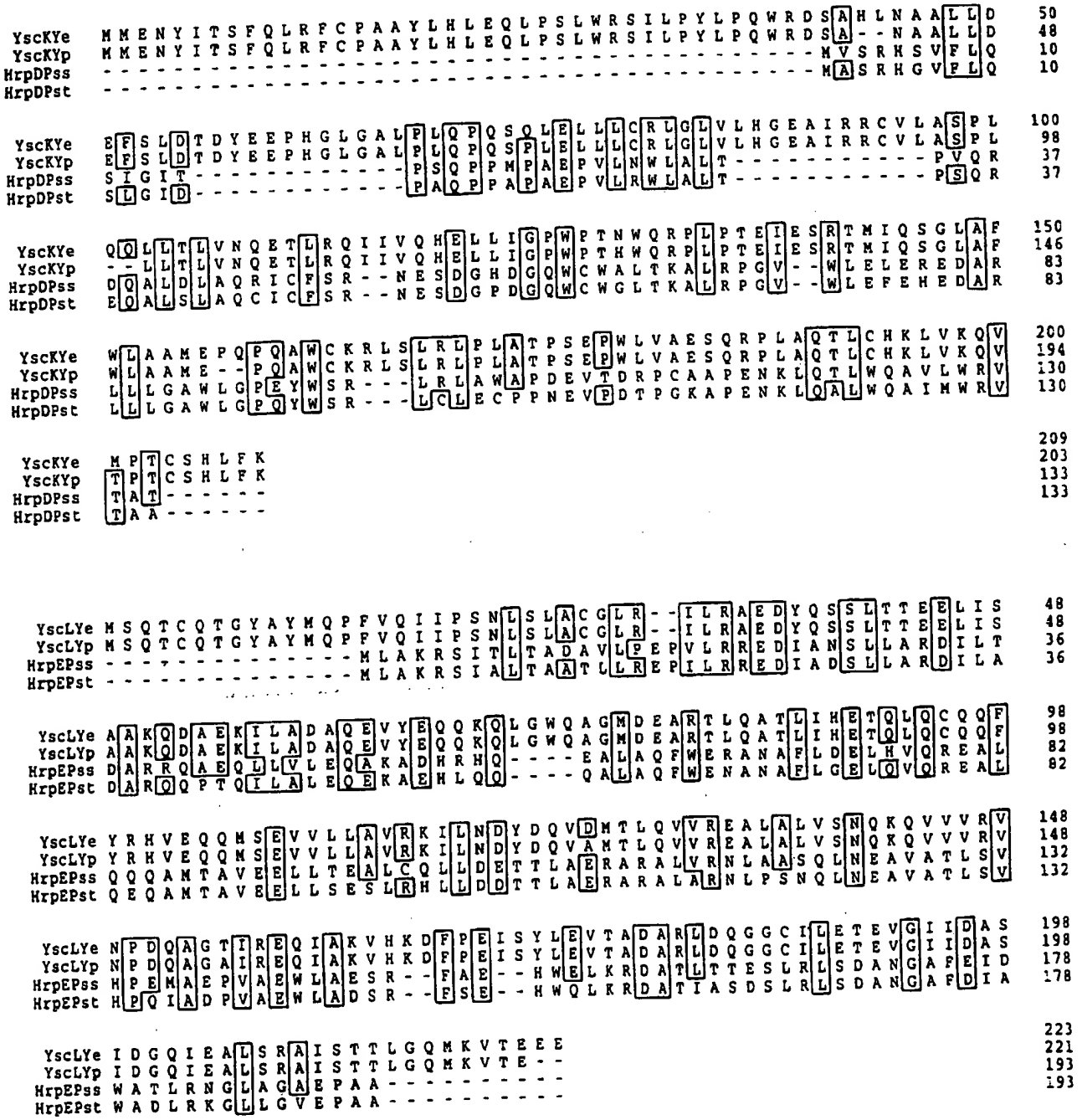

Fig. 6. *(continued from preceding page)*

nificant proportion of the *virC* operon is conserved in *P. syringae*, albeit in a rearranged form. Eckhardt (1978) gels of total DNA, Southern-blotted and probed with a 0.75-kb *Bst*XI internal fragment of $hrpZ_{Pss}$, suggested that the *hrp* genes are chromosomal in the three strains of *P. syringae* studied, rather than being plasmid-borne as are the *hrp* genes of *P. solanacearum* GMI1000 or the *ysc* genes of *Yersinia* spp. (Van Gijsegem et al. 1993; data not shown). The homologies of the *hrpZ* operons are summarized in Table 1.

Overexpression, purification, and biological assay of $HrpZ_{Pst}$ and $HrpZ_{Psg}$

Partially purified lysates of *E. coli* expressing $HrpZ_{Pst}$ and $HrpZ_{Psg}$ elicited a clear HR on tobacco while control lysates of *E. coli* containing vector alone did not. However the activity of the cell lysates on the two host plants was more ambiguous. Soybean is generally unreactive to cell lysates from either pathogen, while tomato is quite sensitive and sometimes weakly reactive not only to cell lysates of *E. coli* expressing HrpZ, but also to control lysates of *E. coli* containing vector alone. To accurately evaluate the biological properties of HrpZ from each of the two pathovars, it was necessary to purify HrpZ. It was also necessary to ascertain that the HR observed on tobacco was due solely to HrpZ and not to the products of either of the two flanking ORFs, HrpA and HrpB, since HrpA and a fusion protein of HrpB were being expressed in addition to HrpZ by the original $hrpZ_{Psg}$ and $hrpZ_{Pst}$ clones.

As a first step towards purifying HrpZ, we attempted to increase the level of expression. From the sequence of the *Pst*I clones encoding *hrpZ* it was clear that long stretches of DNA encoding *hrpA* and the 3′ end of *hrpS* (1,144 bp in $hrpZ_{Psg}^{+}$ pCPP2202 and 809 bp in $hrpZ_{Pst}^{+}$ pCPP2203) separated *hrpZ* from the *lac* promoter in pBluescript II. A series of deletions f the 5′ end of the $hrpZ_{Pst}$ clone were constructed using the Erase-a-Base system (Promega), bringing the *lac* promoter within 100 bp of the *hrpZ* initiation codon, and removing *hrpA*. Although cell lysates expressing the deleted clones retained HR eliciting activity, they did not show a substantial increase in gene expression. Searching for an explanation for this behavior we identified a number of potential contributing factors. The first possibility was the presence of a *cis*-acting sequence contained in the 100 bp remaining upstream of $hrpZ_{Pst}$. Using a terminator analysis pr gram we identified a 9-bp inverted repeat located between *hrpA* and *hrpZ* (Fig. 3). Although this repeat lacks the AT-rich sequ nce downstream which is characteristic of many terminators, it is possible that its presence encourages premature transcription termination. Similar repeats, albeit with weaker secondary structure, can be found upstream of $hrpZ_{Pss}$ and $hrpZ_{Psg}$. A second factor contributing specifically to the low expression of $hrpZ_{Pst}$ may be the absence of a strong ribosome binding site. Finally, there could be factors related to the proteins themselves, such as a lack of stability.

To eliminate possible *cis*-acting sequences and to obtain clones of $hrpZ_{Pst}$ and $hrpZ_{Psg}$ that lack *hrpA* and *hrpB*, the *hrpZ* genes from both pathovars were amplified by PCR, directionally cloned into pBluescript II and transformed into *E. coli* DH5α F′*lacI*^{q}. We obtained significantly increased expression of $HrpZ_{Psg}$ using the plasmid pCPP2255 (Fig. 7), but unexpectedly, overexpression of $HrpZ_{Pst}$ appeared to be deleterious to the cells, and plasmids recovered from transformants often showed rearrangements. To maximize expression of $HrpZ_{Pst}$ under these conditions, we introduced subclones containing the gene behind the T7 promoter of pET21(+) (Novagen, Madison, WI). Unlike the *lac* promoter, the T7 promoter is less sensitive to distance effects, and expression of $HrpZ_{Pst}$ in *E. coli* BL21(DE3), with pET21(+) as the vector, resulted in increased expression as shown in Figures 2 and 8. Expression in BL21(DE3) also allowed us to retain almost complete repression of *hrpZ* until induction with IPTG. Good expression of $HrpZ_{Pst}$ was achieved using the plasmid pCPP2211 in *E. coli* BL21(DE3).

The quality of the samples obtained following partial purification of the lysates by heat treatment was quite variable. To ensure removal of the majority of the contaminating proteins and to obtain a more concentrated sample of protein, we further purified HrpZ by ammonium sulphate precipitation and hydrophobic chromatography, which as indicated in Figure 8, yielded a distinct band on a Coomassie-stained gel. Purified, active HrpZ could then be obtained by electroelution fr m excised gel slices. This procedure was also used to isolate

Table 1. Homologies of *Pseudomonas syringae* pv. *syringae hrpZ* operon proteins with proteins from other *P. syringae* pathovars and *Yersinia* spp.

	HrpA (108)[a]	HrpZ (341)	HrpB (124)	HrpC (268)	HrpD (133)[d]	HrpE (193)
P. s. pv. *syringae*			(124)			
P. s. pv. *glycinea*	(108) 91/92[b]	(345) 79/87	94/96			
P. s. pv. *tomato*	(108) 28/42	(370) 63/75	(124) 68/80	(268) 90/95	(133) 78/87	(193) 76/87
Y. enterocolitica			YscI (115) 22/45[c] 24/45	YscJ (244) 35/59 38/60	YscK (203) 26/53 22/48	YscL (223) 21/47 22/46
Y. pseudotuberculosis			(115) 22/45 21/44	(244) 35/59 38/60	(209) 28/57 23/49	(221) 21/47 22/46

[a] Number of amino acids in the protein is given in parentheses.
[b] Percent identical and similar amino acids in comparison with the *P. s.* pv. *syringae* protein.
[c] The first pair of values are the percent identical and similar amino acids in comparison with the *P. s.* pv. *syringae* protein; the second are in comparison with *P. s.* pv. *tomato*.
[d] The data presented here are for the shorter of the two potential ORFs encoding *hrpD*. The larger versions of the HrpD proteins of *P. s.* pv. *syringae* and *P. s.* pv. *tomato* would be respectively 175 and 176 amino acids long with 74/84% identity/similarity to each other.

HrpZ from the supernatants of *P. s.* pv. *tomato* and *P. s.* pv *glycinea* grown in *hrp*-inducing minimal media (Fig. 9). Preparations of the purified HrpZ proteins from *P. s.* pvs. *syringae*, *glycinea*, and *tomato*, at a concentration f ≥20 μM in MES buffer, were infiltrated into the leaves of tobacco, soybean, and tomato. The three proteins elicited a collapse involving >50% of the infiltrated tissue in tobacco and tomato leaves that developed within 18 h and was typical of the HR elicited by incompatible *P. syringae* strains, but they caused no visible reaction in soybean. It is worth noting that tobacco and tomato plants vary substantially in their sensitivity to harpin preparations. For example, some leaves on sensitive tomato plants will respond to 2 to 5 μM $HrpZ_{Pst}$, but ≥20 μM is required for consistent results. Furthermore, unlike tobacco, tomato plants that have responded hypersensitively to a HrpZ preparation do not respond to subsequent infiltrations of the elicitor. The spurious necroses sometimes observed were deduced to result from mechanical damage incurred during infiltration or the infiltration of preparations contaminated with salts or containing high concentrations of vector control *E. coli* lysates. These necroses developed much more quickly (within 4 to 6 h), and were much weaker and patchier than the confluent HR elicited by HrpZ. The fact that the HR induced by HrpZ in tomato and tobacco is an active response of host tissue was confirmed by coinfiltration of either sodium vanadate at $5^{-5} \times 10^{-3}$ M or lanthanum chloride at 1 × 10 M. Each of these two inhibitors of plant metabolism completely inhibited the HR elicited by HrpZ preparations from each of the three pathovars but not the necrosis caused by the other factors mentioned.

DISCUSSION

We have used the *P. s.* pv. *syringae* 61 *hrpZ* gene to isolate the *hrpZ* locus from *P. s.* pv. *glycinea* race 4 and *P. s.* pv. *tomato* DC3000. Characterization of the *hrpZ* genes, products, and flanking DNA of these three pathovars has revealed the structure of the *hrpZ* operon, the relative variation among ORFs within the operon, the presence of genes d wnstream of *hrpZ* that are colinear with a block of genes involved with *Yersinia* virulence protein secretion, and the presence in $HrpZ_{Pst}$ of a sequence related t a sequence in the PopA1

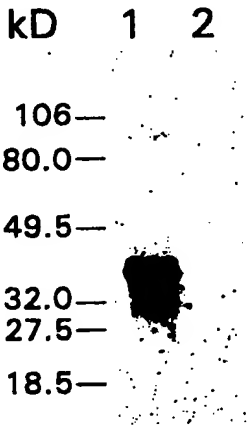

Fig. 7. Overexpression of $HrpZ_{Pst}$ in *E. coli* DH5α F′*lacI*^{Q}. Cultures were grown overnight at 30°C in LM with 1 mM IPTG. Cell lysates were partially purified by heat treatment, separated on an SDS-polyacrylamide gel, transferred to Immobilon-P, immunoblotted with anti-$HrpZ_{Pst}$ antibodies, and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, *E. coli* DH5α F′*lacI*^{Q} (pCPP2255); 2 *E. coli* DH5α F′*lacI*^{Q} (pBluescript II).

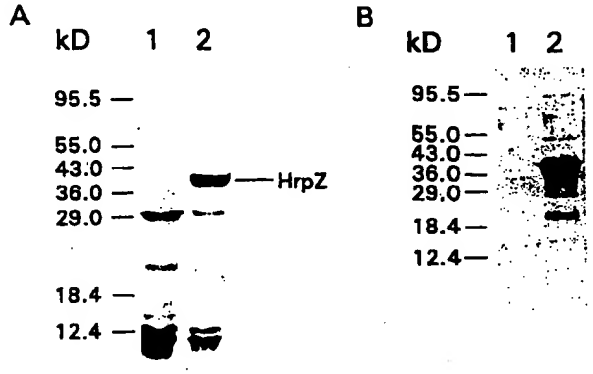

Fig. 8. Overexpression and purification of $HrpZ_{Pst}$. Cultures were grown to an OD_{600} of 0.6 and induced with 1 mM IPTG. $HrpZ_{Pst}$ was then partially purified from the cell lysate in a three-step process: first, by heat-treatment at 100°C as previously described, then by precipitation with ammonium sulphate at 30 to 45% saturation, and finally by binding to a hydrophobic resin (phenyl-sepharose) at 30% ammonium sulphate. A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, *E. coli* BL21(DE3)(pET21+); 2, *E. coli* BL21(DE3)(pCPP2211). B, Immunoblot of the samples shown in A, probed with anti-$HrpZ_{Pst}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.

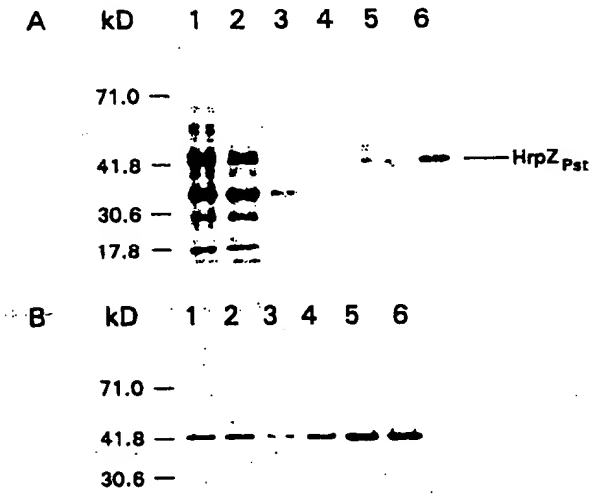

Fig. 9. Purification of $HrpZ_{Pst}$ from *hrp*-induced *Pseudomonas syringae* pv. *tomato*. Cells were grown in King's broth (KB) at 30°C and then resuspended in *hrp*-inducing minimal medium (Huynh et al. 1989) and incubated at room temperature overnight. Cells were removed by centrifugation and the supernatant heat-treated at 100°C for 10 min. Proteins in the supernatant were precipitated with ammonium sulphate at the percent saturations indicated. Proteins were desalted, concentrated, and resuspended in 5 mM MES using Centricon-10 tubes (Amicon). A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, supernatant extracted with Strataclean resin (Stratagene); 2, heat-treated supernatant extracted with Strataclean resin (Stratagene); 3, 0 to 20% ammonium sulphate fraction; 4, 20 to 30% ammonium sulphate; 5, 30 to 40% ammonium sulphate; 6, 30 to 45% ammonium sulphate. B, Immunoblot of the samples shown in A, probed with anti-$HrpZ_{Pst}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.

protein of the tomato pathogen *P. solanacearum* GMI1000. We also observed that purified $HrpZ_{Pss}$ was at least as effective as $HrpZ_{Pss}$ and $HrpZ_{Psg}$ in eliciting an HR-like necrosis in the leaves of tomato, a host of *P. s.* pv. *tomato* DC3000, whereas none of the HrpZ preparations elicited significant necrosis in soybean, the host of *P. s.* pv. *glycinea*.

The HrpZ proteins of three *P. syringae* pathovars.

A comparison of the sequences of the three HrpZ proteins with each other and with HR elicitors characterized from other bacteria indicates that the HrpZ proteins represent a distinct family of elicitors that is conserved among *P. syringae* pathovars. The amino acid sequences of the three proteins are sufficiently similar to reveal their relatedness, but (with the exception of a sequence within $HrpZ_{Pss}$), they show no significant relatedness to elicitor proteins from other bacteria. Interestingly, *hrpZ* is the second most divergent ORF in the *hrpZ* operons of *P. s.* pv. *syringae* and *P. s.* pv. *tomato*, with only 63% of the predicted amino acids being identical. Nevertheless, $HrpZ_{Pss}$, $HrpZ_{Psg}$, and $HrpZ_{Pss}$ are indistinguishable in several biological and physical properties. They have the same effect on different plants (discussed below), and they are heat stable, glycine-rich, and devoid of cysteine and tyrosine. The lack of tyrosine is a feature they differentially share with the *P. solanacearum* PopA1 protein but not the *Erwinia* harpins. This property has been speculated to allow the protein to avoid the H_2O_2-mediated cross-linking of tyrosine residues that may occur in plant cell walls during defense responses (Bradley et al. 1992; He et al. 1993).

Interestingly, a 24 amino acid, glycine-rich stretch of $HrpZ_{Pss}$ shows homology to part of PopA1, as does the cognate nucleotide sequence. The region of homology between $HrpZ_{Pss}$ and PopA1 corresponds exactly to the insertion in $HrpZ_{Pss}$. The insertion of this element within $HrpZ_{Pss}$ sequences that are otherwise similar among the three HrpZ proteins suggests horizontal transfer and a common ancestry with PopA1. Because the host range of *P. solanacearum* overlaps with that of *P. s.* pv. *tomato*, it is tempting to speculate that this region has some particular significance to pathogenesis on tomato, although, as discussed below, this is not obvious from the different effects of the two proteins on tomato.

The presence of this insert in active $HrpZ_{Pss}$ is another indicator of the apparent plasticity of structure/function relationships in these glycine-rich elicitor proteins. That significant changes to the structure of these proteins does not abolish their activity was previously demonstrated when a fortuitous $hrpZ_{Pss}$ clone was found to produce an active derivative of HrpZ missing the N-terminal 125 amino acids, and the *popA* product was observed to be degraded in culture to an active form missing the N-terminal 93 amino acids (He et al. 1993; Arlat et al. 1994). Clearly the presence of this "additional" internal sequence does not diminish the ability of the protein to elicit the HR. In fact, although it is difficult to make a quantitative assessment, $HrpZ_{Pss}$ may actually be a slightly more potent elicitor of the HR than $HrpZ_{Pss}$.

However, $HrpZ_{Pss}$ appears to differ from the other HrpZ proteins in being deleterious to *E. coli* cells when overexpressed and is possibly more unstable, making it difficult to purify large amounts of the protein. Since the glycine-rich region is the most obvious difference between $HrpZ_{Pss}$ and $HrpZ_{Pss}$ it is possible that it contributes to this phenomenon. We were able to overcome this problem experimentally by using a tightly regulated T7 promoter/polymerase system, but never obtained quite the same level of expression we achieved with $HrpZ_{Pss}$ and $HrpZ_{Psg}$. However, there remains the obvious question of how HrpZ toxicity is avoided by *P. s.* pv. *tomato*. One possibility would be that HrpZ is never expressed at levels high enough to affect the bacterium, even when it is induced in planta. Some indirect evidence for this hypothesis is provided by our examination of the DNA upstream of $hrpZ_{Pss}$. The ORF has a weak ribosome binding site, and we also observed that expression of cloned *hrpZ* from the *lac* promoter appears to be attenuated by the presence of *cis*-acting upstream sequences. A 9-bp GC-rich repeat upstream of *hrpZ* may be significant in this regard. Preliminary data from northern blotting experiments also indicate that premature transcription termination may take place when *hrpA-hrpZ* clones are expressed in *E. coli* (G. Preston, unpublished). A second possibility is that the location of the *hrpZ* gene in an operon with secretion genes ensures tight coupling of synthesis and secretion. Genes encoding extracellular proteins and secretion pathway components are often coregulated, but with a few exceptions involving the type I pathway, they do not lie within the same operon (Fath and Kolter 1993). A third possibility is that *P. s.* pv. *tomato* is more tolerant of high levels of HrpZ than is *E. coli*, or it possesses a means of keeping HrpZ in a nontoxic form while it is in the cell.

Further comparison with the *Yersinia* virulence system presents an intriguing possibility in this regard. It has been shown that secretion of certain "Yops" (the *Yersinia* pathogenicity determinants), involves chaperone proteins, small hydrophilic proteins which help keep the Yop protein in a translocation competent form and help target it for secretion (Wattiau et al. 1994). The genes encoding each chaperone are located adjacent to the gene encoding the corresponding Yop. Given the presence of several small ORFs of undetermined function in the pHIR11 *hrp* cluster, it is tempting to speculate that one of them, particularly *hrpA*, might encode a protein with chaperone function. There is a superficial resemblance between HrpA and *Yersinia* chaperones such as SycE. They are all small, hydrophilic, cytoplasmic proteins which lack a signal sequence, but there are no specific homologies. We are now constructing nonpolar mutations to test the role of HrpA in secretion. Preliminary results suggest that HrpA is not required for *E. coli* MC4100(pHIR11) to elicit an HR or secrete HrpZ (J. R. Alfano, unpublished), but in chaperone-mediated systems limited secretion of a protein will usually occur even in the absence of its chaperone, so it may be necessary to look quantitatively at secretion and accumulation of HrpZ to assess whether mutations in *hrpA* or other *hrp* genes have an effect.

The colinear relationship between several *hrp* and *ysc* genes.

From the sequence of the *hrpZ* operon it is clear that the parallels with the *Yersinia* type III secretion pathway extend bey nd homologies of individual genes. The four genes downstream of *hrpZ*, *hrpB–E*, appear to be arranged colinearly with the region of the *virC* secretion operon from *Yersinia* that encodes YscI–L. The *virC* peron is a large operon containing 13 genes, *yscA-yscM*, several f which have been demonstrated to have a role in Yop secretion (Michiels et al. 1991). Of the four *Yersinia* genes with putative ho-

mologs in the *hrpZ* operon, only *yscJ* and *yscL* are known to have a role in secretion. An accompanying paper shows that five more *hrp* genes, downstream of the *hrpH* operon, are colinear with the *yscQ–U* genes in the *virB* operon of *Yersinia* (Huang et al. 1995).

It appears that a significant proportion of the type III secretion pathway described in *Yersinia* can be identified in *P. syringae*, and it seems likely that increasing parallels between the two systems will be found. In both systems the secreted proteins are involved with early events in the interaction with the host, and expression of secretion genes and virulence proteins is tightly coregulated. The secretion pathway seems to function in a similar way, as in both cases secreted proteins lack an N-terminal signal peptide and are not posttranslationally processed.

HrpZ and host specificity.

The function of HrpZ in compatible interactions is unclear. A likely role is the release of nutrients to the apoplast. Atkinson and Baker (1987a, 1987b) have proposed that the alkalinization of the apoplast caused by Hrp$^+$ bacteria (which occurs at a slower rate in compatible interactions) results in the leakage of sucrose and other nutrients to support bacterial growth. One of the key unanswered questions regarding the *P. syringae* HrpZ proteins is their role in host specificity. Compatible interactions leading to disease are distinguished by the absence of the HR. Host-differential elicitor activity would be one way to reconcile the production of HR-eliciting proteins by *P. syringae* and the phenomenon of host-specific compatibility. The failure of the PopA1 protein to elicit the HR in tomato, a host of *P. solanacearum* GMI1000, supports this concept (Arlat et al. 1994). Similarly, the isolated *P. s.* pv. *syringae* 61 HrpZ protein fails to elicit the HR in bean, although the significance of this is diminished by the fact that bean leaves appear insensitive to any harpins (He et al. 1993). To further explore this question, we infiltrated all three HrpZ proteins into the leaves of the host plants for each of the pathovars. The host plants of *P. s.* pv. *syringae* 61, and *P. s.* pv. *glycinea*, bean and soybean, respectively, are uniformly unreactive to HrpZ from both compatible and incompatible pathogens; however, tomato leaves proved to be highly sensitive to all three HrpZ proteins. Thus, our data argue against the hypothesis that host-differential activity of HrpZ proteins controls the host specificity of *P. syringae* pathovars.

If isolated $HrpZ_{Psi}$ elicits the HR in tomato, why does *P. s.* pv. *tomato* not elicit the HR during pathogenesis? One possibility is that the response of tomato to $HrpZ_{Psi}$ is qualitatively different than the response to $HrpZ_{Psa}$ and $HrpZ_{Pss}$ despite manifestation of the same gross morphology. That is, the necrosis elicited by $HrpZ_{Psi}$ is fundamentally different than the HR and does not involve associated defenses that stop the pathogen. We are now testing this possibility with probes for HR-specific transcripts. A second possibility is that $HrpZ_{Psi}$ production is regulated in a host-specific manner. However, *hrpZ* is clearly part of the Hrp regulon: *hrpZ* expression is transcriptionally linked with genes encoding components of the secretion pathway, the *hrpZ* operons in all three of these *P. syringae* pathovars have virtually the same *hrp/avr* promoter sequence, and expression of the *hrpZ* operon is likely required for pathogenicity. The conserved promoter sequences suggests that the *hrpZ* operon is regulated in *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* by the same nutritional conditi ns and HrpR, HrpS, HrpL regulatory cascade described for *P. s.* pv. *syringae* and *P. s.* pv. *phaseolicola* (Grimm and Panopoulos 1989; Rahme et al. 1992; Xiao et al. 1992; Xiao et al. 1994; Xiao and Hutcheson 1994; Grimm et al. 1995). Whether differential expression of the Hrp regulon controls host specificity awaits determination. A third possibility is that the *P. syringae* pathovars produce host-specific suppressors of defense responses. This is supported by the observation that compatible pathogens do not trigger defense responses in host plants that are elicited by nonpathogens (Jakobek et al. 1993).

It is important to note that our data do not eliminate the possibility that the three HrpZ proteins actually have differential activity in host plants when delivered by living bacteria and that the HR observed may be an abnormal response resulting from the presentation of a high concentration of HrpZ in an artificial manner. In that regard, it is interesting that legumes, which appear insensitive to isolated harpins, respond to Hrp recombinant *E. coli* cells that secrete the same proteins (He et al. 1993). Experiments in which the *hrpZ* genes of *P. syringae* pathovars are switched or altered in their patterns of deployment should test more definitively the role of HrpZ in determining host specificity.

In conclusion, we have characterized an operon containing two components of the Hrp$^+$ system of *P. syringae*—a block of secretion-related genes that are conserved in eukaryotic pathogens in the genera *Pseudomonas*, *Xanthononas*, *Erwinia*, *Yersina*, *Shigella*, and *Salmonella* and a gene encoding an elicitor that is unique to plant pathogens. The elicitors found in the *P. syringae* pathovars are a subfamily of a larger class that appears to be characteristic of plant pathogens, and which we postulate to have a role in releasing nutrients for bacterial utilization. Our challenge now is to determine how the various components of the Hrp system have been adapted to serve plant parasitism in the face of plant defenses.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacteria and plasmids used in this study are shown in Table 2.

Pseudomonads were routinely grown in King's B broth (King et al. 1954) at 30°C, but for certain experiments the *hrp*-derepressing minimal medium of Huynh et al. (1989), adjusted to pH 5.5, was used. *E. coli* was grown in LM (Sambrook et al. 1989) or terrific broth (Tartof and Hobbs 1987). Plasmids were introduced into bacteria by transformation (Sambrook et al. 1989) or electroporation (Gene Pulser, Bio-Rad).

Plant materials.

The plants used in this study were tobacco (*Nicotiana tabacum* L. 'Xanthii'), tomato (*Lycopersicon esculentum* Mill. 'Moneymaker'), and soybean (*Glycine max* L. 'Harosoy'). Plants were grown in a greenhouse or growth chamber at 23° to 25°C with a photoperiod of 16 to 24 h. Infiltration of plant leaves with HrpZ preparations was performed with blunt syringes as described (Huang et al. 1988).

DNA analysis and sequencing.

All DNA manipulations, except where specified, followed standard protocols (Ausubel et al. 1987; Sambrook et al. 1989). The *hrpZ* region of pHIR11 was subcloned into

pBluescript II (Huang et al. 1995). Two *Pst*I fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200, respectively, were subcloned into pBluescript II SK(–) in both orientations. A series of overlapping nested deletions covering both strands was generated for each of the subclones using Erase-a-Base (Promega, Madison, WI). The deletions were sequenced from double-stranded templates using Sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH) and forward and reverse M13 primers. Sequencing was completed using specific primers synthesized by Integrated DNA Technologies (Coralville, IA). In addition, the 3.7 and 3.6 kb *Sac*I-*Eco*RI fragments, which overlap the *Pst*I subclones from pCPP2201 and pCPP2200, were also subcloned into pBluescript II SK(–) and sequenced using the ABI 373A DNA sequencer at the Cornell Biotechnology Program DNA sequencing facility and specific primers synthesized by IDT. Nucleotide and derived amino acid sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux et al. 1984). Homology searches against major sequence databases were done with the BLAST program (Altschul et al. 1990).

PCR amplification of *hrpZ* from *P. s.* pv. *glycinea* and *P. s.* pv. *tomato*.

The *hrpZ* genes of *P. s.* pv. *glycinea* and *P. s.* pv. *tomato* were amplified by PCR from the plasmids pCPP2202 and pCPP2203, respectively. Reactions were performed using the PCR Optimizer kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Reactions were overlaid with mineral oil and incubated in a Hybaid Thermal Reactor (Hybaid, Teddington, U.K.) using these cycle parameters: 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, followed by a final incubation of 7 min at 72°C. The primers used for $hrpZ_{Psg}$ were 5′-TACGGGATCCTTTGAGGAGGTTGTGATG-3′ and 5′-TACGCTGCAGTATC AGTCAGGCAGCAGC-3′, and those for $hrpZ_{Psi}$ were 5′-TACGGGATCCATGCAAGCACTTA ACAGC-3′ and 5′-GGAACTGCAGCAAGCTCCGGCGA-TACAC-3′. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and were designed to introduce a *Bam*HI and a *Pst*I site at the 5′ and 3′ ends, respectively, of each amplified fragment.

The $hrpZ_{Psg}$ fragment from pCPP2202 was successfully amplified in all reaction buffers tested. The $hrpZ_{Psi}$ fragment from pCPP2203 was successfully amplified using reaction buffer B (reaction concentration 60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, pH 8.5). PCR products of the expected sizes of 1.0 and 1.2 kb were purified from an agarose gel, digested with *Pst*I and *Bam*HI, cloned into pBluescript II, and then transformed into *E. coli* DH5α F′*lac*I, yielding plasmid pCPP2255 carrying $hrpZ_{Psg}$. Plasmids containing

Table 2. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Reference or source
Escherichia coli		
DH5α	*sup*E44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 Nal^r	Hanahan 1983; Life Technologies, Inc. Grand Island, NY
DH5α F′*lacI*^{q}	F′ *pro*AB$^+$ *lacI*^{q}ZΔM15 zzf::Tn5[Km^r]/ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *end*A1 *rec*A1 *hsd*R17 ($r_k^-m_k^+$) *deo*R *thi*-1 *sup*E44λ$^-$ *gyr*A96 *rel*A1	Life Technologies Inc.
BL21(DE3)	F′ *omp*T *hsd*B_B($r_B^-m_B^-$) *dcm gal* DE3	Novagen
Pseudomonas syringae		Baker *et al.* 1987
pv. *syringae* 61	Wild type	C. J. Baker
pv. *glycinea* race 4	Wild type	D. E. Cuppels
pv. *tomato* DC3000	Wild type, Rp^r	
Plasmids		
pBluescript II SK(-)	Cloning vector, Amp^r	Stratagene
pUCP19	pUC19 derivative, Amp^r	Schweizer 1991
pET21(+)	T7 transcription vector, Amp^r	Novagen
pT7-6	T7 transcription vector; Amp^r	Tabor and Richardson 1988
LITMUS 28	Cloning vector, Amp^r	New England Biolabs
pHIR11	25-kb cosmid containing *P.s.* pv. *syringae* 61 *hrp* cluster	Huang *et al.* 1988
pSYH10	$hrpZ_{Psi}$ ORF in pBluescript II	He *et al.* 1993
pCPP2303	0.8-kb *Pst*I-*Age*I subclone from pHIR11, containing *hrpB*, in LITMUS 28	This study
pCPP2305	1.3-kb *Sal*I-*Sac*I subclone from pHIR11, containing *hrpD*, in pT7-6	This study
pCPP2200	pUCP19 carrying 10-kb partial *Sau*3A1 fragment of *P. s.* pv. *glycinea* DNA with $hrpZ_{Psg}$	This study
pCPP2202	2.4-kb *Pst*I subclone of pCPP2200 in pBluescript II; $hrpA_{Psg}$ and $hrpZ_{Psg}$ in expressed orientation with respect to P_{lac}	This study
pCPP2204	As pCPP2202 but with $hrpZ_{Psg}$ in reversed orientation to P_{lac}	This study
pCPP2206	2.4-kb *Pst*I $hrpA_{Psg}$ and $hrpZ_{Psg}$ subclone from pCPP2202 in pET21(+)	This study
pCPP2208	3.6-kb *Sac*I-*Eco*RI $hrpZ_{Psg}$ subclone from pCPP2200 in pBluescript II	This study
pCPP2210	1.85-kb *Bgl*II-*Pst*I $hrpZ_{Psg}$ subclone from pCPP2202 in pET21(+)	This study
pCPP2255	PCR-amplified $hrpZ_{Psg}$ ORF in pBluescript II	This study
pCPP2201	pUCP19 carrying 10-kb fragment of *P. s.* pv. *tomato* DNA with $hrpZ_{Psi}$	This study
pCPP2203	2.2-kb *Pst*I subclone of pCPP2201 in pBluescript II; $hrpA_{Psi}$ and $hrpZ_{Psi}$ in expressed orientation with respect to P_{lac}	This study
pCPP2205	As pCPP2203 but with $hrpZ_{Psi}$ in reversed orientation to P_{lac}	This study
pCPP2207	2.2-kb $hrpZ_{Psi}$ subclone from pCPP2203 in pET21(+)	This study
pCPP2209	3.7-kb *Sac*I-*Eco*RI subclone from pCPP2201 containing $hrpBCDE_{Psi}$ in pBluescript II	This study
pCPP2304	3.7-kb *Sac*I-*Eco*RI subclone from pCPP2209 in LITMUS 28	This study
pCPP2211	2.0-kb *Bgl*II-*Pst*I $hrpZ_{Psi}$ subclone from pCPP2203 in pET21(+)	This study

[a] Amp^r = ampicillin resistance; Nal^r = nalidixic acid resistance; Rp^r = rifampicin resistance.

PCR-amplified $hrpZ_{Pst}$ were found to be unstable and appeared to promote cell lysis.

HrpZ purificati n and analysis.

HrpZ was purified from *E. coli* as previously described (He et al. 1993) with the following modifications. Cells were lysed in either 5 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5, or cell lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). For some experiments the supernatant from heat-treated lysate was partially purified after sonication by ammonium sulphate precipitation (25 to 45% saturation), with desalting and concentration being performed with Centricon-10 tubes (Amicon). For experiments requiring highly purified HrpZ expressed in *E. coli* BL21(DE3), the supernatant was further purified by binding to phenyl-sepharose (Sigma) in the presence of ammonium sulphate (>30% saturation) and elution with 5 mM MES, pH 5.5, followed by electrophoresis through a native 15% polyacrylamide gel. The purified protein was then eluted from excised gel slices using an Elutrap apparatus (Schleicher & Schuell) or from crushed gel slices using a Micropure separator (Amicon). Protein concentrations were determined using Bio-Rad protein assay solution. HrpZ was also purified from heat-treated supernatants of *P. syringae* grown in *hrp*-inducing medium (Huynh et al. 1989) by ammonium sulphate precipitation (25 to 45% saturation) and desalting/concentration using Centricon-10 tubes. For infiltration into plant tissue, HrpZ preparations were diluted to various degrees with 5mM MES, pH 5.5. The amino-terminal sequence analyses were performed at the Cornell Biotechnology Program Protein Analysis Facility ($HrpZ_{Psg}$) and the University of Kentucky Macromolecule Structure Analysis Facility ($HrpZ_{Pst}$).

T7 expression and labeling of proteins in *E. coli*.

Proteins encoded by the *hrpZ* operon were expressed in *E. coli* BL21(DE3) by using the pET21(+) T7 expression system (Novagen). Conditions for isopropyl-β-D-thiogalactopyranoside (IPTG) induction of T7 RNA polymerase-dependent expression and labeling with L-[^{35}S]methionine were as described by Studier et al. (1990). After being labeled, cells were collected by centrifugation and then resuspended and lysed in SDS-loading buffer and the proteins resolved on an SDS-polyacrylamide gel. Gels were stained, dried and exposed to Kodak X-ray film.

Nucleotide sequence accession numbers.

The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers L41861 (*P. syringae* pv. *tomato hrpA, hrpZ, hrpB, hrpC, hrpD, hrpE*). L41862 (*P. syringae* pv. *glycinea hrpA, hrpZ, hrpB*), L41863 (*P. syringae* pv. *syringae hrpA*), and L41864 (*P. syringae* pv. *syringae hrpB*).

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